Bridging gaps in cardiac repair; Looking back to think ahead

Het overbruggen van kennishiaten in cardiale regeneratieve therapie; terugkijken om vooruit te denken

Proefschrift

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Prelude & General introduction

Prelude

Diseases and potential new cures make news headlines daily. A search on Google Trends, a tool tracking search trends on Google, reveals that both 'cell therapy' and 'heart disease' are on the top of many minds in the civilized world.[1,2] This is not surprising, as acute cardiac diseases are better treated and maintained, but chronic cardiac care is increasing for heart failure patients either due to overcome acute ischemic events or other acquired/hereditary cardiomyopathies.
Still 17.5 million people die yearly from cardiovascular disease worldwide, of which 80% can be attributed to heart attacks and stroke.[3] To put these numbers in cruel perspective; that is the entire population of the Netherlands, gone, in a year.

Myocardial infarction does not only cause mortality but also serious health complications. Ischemic heart disease is one of the main contributors to the heart failure epidemic we are currently facing. Both in terms of morbidity and costs, the numbers are growing as we speak.[4] As people are surviving ischemic events, growing older and are increasingly becoming obese and diabetic, it makes perfect sense that these numbers are on the rise.[4] In the United States alone, medical care costs for heart failure will likely triple by 2030 and the prevalence of heart failure will increase at an even faster rate.[4]

One of the suggested options that might lead to a solution for 'heart disease' is 'cell therapy'. Taking advantage of additional Google Trends' features, it lets one see related subjects to any search query. It was not entirely surprising, but disappointing nonetheless, that in data from the last 5 years the search term 'cell therapy' was not nearly combined as much to 'heart disease' as it was to 'hair transplants'. We firmly believe that cell therapy in combination with heart disease deserves more of the general spotlight, both in our scientific community and the general public. If you are reading this thesis and have one minute to spare, please type in https://www.google.nl/#q=cell+therapy+AND+heart+disease, or click the link in the pdf to get our subject on top of Google Trends!

Introduction

Roadmaps to new cardiovascular therapeutics

The process of scientific research weighs substantially on industry, governments, non-governmental organizations and universities. In the pursuit of new therapies and ultimately cures for many diseases, we invest billions in different stages of therapeutic research. This usually happens according to a mandatory roadmap (Figure 1).

The search for new therapeutics usually starts in the lab, where new processes and signaling pathways are identified on a cellular level (*in vitro)*, which are sometimes based on clinical clues and data. If a process seems to have 'therapeutic added value' in for example heart disease, and is 'druggable' (meaning we can interfere with the pathway to either turn it off or on), this will be shown in the lab *in vitro* before we move forward. The next step is an animal experiment (*in vivo)*, in which a disease of interest is mimicked in animals and the new therapy is compared to animals receiving a placebo treatment. This is usually tested in rodent models first, because these are the most cost-effective to maintain, allows for best reproducibility using inbred strains and can be genetically modified to mimic specific diseases or risk factors.[5] In the last preclinical phase, researchers switch to larger animal models like rabbits, dogs, sheep and pigs, as these come closer to real-life human size and physiology.[6] Researchers are also distinguishing animal studies on their goal of being exploratory in terms of biological mechanisms or confirmatory in terms of clinical translatability.

If these steps are completed and the therapy seems safe, feasible and efficacious in these animal models, human trials can be commenced if approved by regulatory bodies. If such a therapy passes the phases of all clinical trials and proves to have an additional clinical benefit, it will be added to guidelines and standard care.

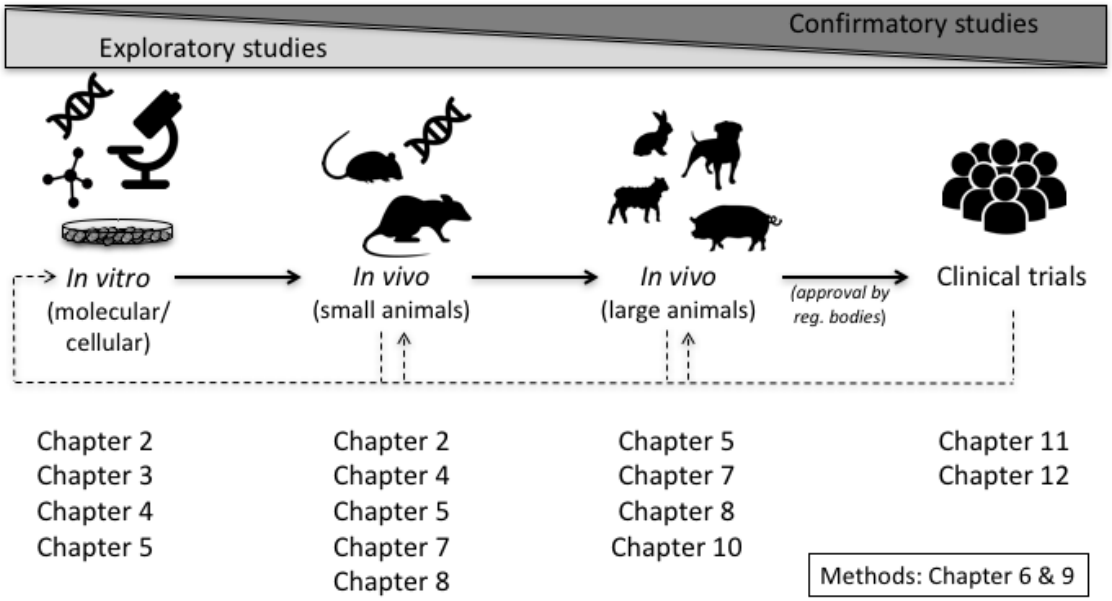

Figure 1. Common mandatory roadmap for new therapeutics

Translational failure

The steps that are taken from the initial discovery to the standard implementation in the clinic can take many decades. The system has many quality controls in place to ensure findings are true and meaningful in the early stages of research. Because of these checkpoints, many new

therapeutics rightfully fail to reach the shelf, let alone a clinical stage, and fail to show an added effect in any of the stages depicted in Figure 1. Reproducibility of preclinical research seems limited, increasing skepticism nowadays for conducted high-impact animal research.[7,8]
On top of these drawbacks, additional failures to clinically translate a drug can be attributed to multiple reasons. Among other reasons, the drug can have serious side effects in humans, a reduced or different biological mechanism in the human setting or doesn't show the same efficacy as it did in the preclinical phase for (partially) unknown reasons. The latter intangible 'translational failure' is especially more the rule than the exception in current biomedical research. There are multiple factors at play that all influence this process, of which heterogenic disease spectra, validity of studies and certain biases are major components.[9]

Internal and external validity
When assessing the value of any study, we are talking about different forms of 'validity'. Internal validity is the extent to which the observed effects can be attributed to the treatment under study. If all else is controlled for and a proper power calculation has been conducted, besides random chance there should be only one reason for the difference between two groups: the intervention under study.
External validity is the extent to which the observed effects in a study can be translated to the (usually clinical) situation that was modeled. If the disease model accurately resembles the clinical disease, this should always be the case. However, disease models are artificial and usually oversimplified, while true diseases are heterogeneous, multimodal and dependent on multiple environmental cues and risk factors.[10] Incorporation of these risk factors is being proposed, which have shown validating results for treatments, but also different efficacies when risk factors like hypertension or diabetes are incorporated.[11]

Biases
Internal validity can be affected by many biases. *Selection bias* can occur at the earliest stages of disease, selectively allocating specific animals to control or therapy group. *Performance bias* relates to the care subjects are getting throughout the study, which might affect outcomes if these differ between treatment groups. *Detection bias* is the phenomenon in which the outcome assessor is aware of the group the observed subject is in, therefore (sub)consciously 'expecting' a certain result from an animal. The selective inclusion and exclusion of animals or other unequal deviations from the protocol is called *attrition bias* and can also affect the primary outcome of studies.[12] The good thing about all these biases affecting internal validity is that they can be almost completely eradicated by the use of blinding and randomization. *Publication bias* is referred to as the selective publishing of merely positive studies, which will cause an unrealistic efficacy expectation for future endeavors, therefore affecting external validity.[13]
Educating young researchers is key in these, so that they will be aware of, recognize and prevent certain biases and errors. Of note, not every study can and should be completely blinded and randomized, but if one wants to confirm the real effect of a given intervention one needs to exercise utmost care to blind and randomize.

<u>Systematic review to assess study quality and more</u>
To accurately measure the quality of evidence in a research field and the presence of biases, one ideally wants to assemble all available knowledge on the subject. Systematic review and meta-

analyses (MA) are tools to systematically assess all available evidence on a subject and synthesize this info into a crucial quality-check and potential other assessments. While already being regularly performed on clinical data, the last decade has seen a rise of systematic review and MA of preclinical studies.[14]
Preclinical studies tend to be somewhat smaller than clinical trials and more prone to some biases and false claims. Furthermore, heterogeneity between preclinical studies is thought to be larger, being both a blessing and a curse; comparability might be lower between studies, while exploring heterogeneity and confirming differences between certain variables of interest might be easier. Interestingly, preclinical MA are less prone to *ecological bias* or *aggregation bias*, one of the common fallacies of clinical MA. It represents the phenomenon that individual outcomes and characteristics cannot always be directly derived from the group's average. As certain characteristics in preclinical MA are kept as constant as possible (to induce the least amount of variation), mean values in preclinical MA's can be better regarded as representing the individuals in certain groups and a real weighted average for the study and variable of interest.
In this thesis, we take preclinical MA and its methodology to the fields of cardiac ischemic disease and cell therapy, showing novel insights through known and novel methodology.

Ischemic heart disease

In this thesis, we focus on ischemic heart disease. This disease is characterized by damage to the myocardium due to a sudden or chronic cardiac oxygen mismatch, usually through insufficient oxygen supply from the coronary arteries. In the case of acute myocardial infarction (MI), the sudden decrease in blood flow usually is restored through percutaneous interventions, which results in the reintroduction of sufficient oxygen levels. This unfortunately also causes the sudden increase of reactive oxygen species and an inflammatory response from the innate immune system, further damaging the heart.[15] As a result of these processes, the heart muscle dies off, being replaced by other cell types like fibroblasts, which proliferate and 'repair' the region with the deposition of extracellular matrix.[16] This process transforms a once contracting muscle in a scar that is usually capable of keeping the form of the heart intact, without aiding in the duty of the cardiac muscle.[16] The process of readapting to this new situation is called 'cardiac remodeling'. If the heart is not able to find a healthy balance between physiologic demand and supply, it will start to hypertrophy or dilate, resulting in 'adverse remodeling' and ultimately heart failure.[16] Unfortunately, the adult heart has a limited capability of regenerating itself after extensive damage.[17] Although cardiomyocytes still divide during normal lifespan[17,18] and might do so increasingly upon hypoxia and other stimulations[19-21], these processes seem futile after the loss of billions of cardiomyocytes in ischemic events. Current regenerative approaches focus on either the administration of new cardiac tissue in the form of cells or stimulating the intrinsic mechanisms of cardiomyogenesis to a larger extent than natural processes are doing.

Cell therapy

One of the focuses of the last decade has been a cell therapy approach, infusing certain cell types that are thought to improve cardiac performance by either transdifferentiating into cardiomyocytes or having a paracrine supportive effect.[22] The latter hypothesis still stands nowadays; commonly used cells like bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (MSCs) and adult cardiac stem cells (CSCs) are thought to act primarily through the supportive substances that they excrete.[23]

There are many different types of cells that have been used over the past decades. After the use of myoblasts,[24] researchers switched to BMMNCs, which are obtained through a bone marrow aspiration and can be prepared for re-injection within hours. One of the landmark trials has been the REPAIR-AMI trial, which was one of the first large trials, convincingly showing the benefit of these cells after acute MI.[25] BMMNCs are the most commonly used cell type today for ischemic heart disease, for which MA have repeatedly shown a small, but significant increase in cardiac function compared to control patients.[26,27] A recent individual patient data MA was not able to confirm this trend.[28] The currently recruiting BAMI trial (www.bami-fp7.eu, clinicaltrials.gov #NCT01569178) will hopefully be able to answer remaining questions shortly, as it is the largest trial for BMMNCs after MI with the primary goal to once and for all determine the effect of BMMNC cell therapy on binary endpoints like mortality.

MSCs are a specific subset, typically derived from the bone marrow (0.01-0.001% of cells from the bone marrow are thought to be MSCs), that can also be isolated from many, if not all, tissues like fat and blood.[29] MSCs are expandable, thought to be immune-privileged and have strong paracrine effects, making them an interesting therapeutic in cell therapy.[30] They are, together with CSCs, considered as a next-generation type of cell therapy, with better properties than, for example, the BMMNCs. Recently, many clinical studies have been completed in both MI and heart failure, with most of them showing promising results.[31-37]

CSCs are a cell therapeutic directly isolated from the heart. There are multiple types of CSCs, recognized by a specific marker like, c-kit, Sca-1, Islet-1 or having a specific isolation procedure like the cardiospheres and side population cells.[38-43] Many of these cell types seem to share similar transcriptional profiles and potential modes of action, despite different isolation strategies.[44] Their residence in the heart and cardiac lineage commitment seem to make them an excellent choice for cardiac cell therapy, although their mechanism is also thought to be mainly paracrine. Their superiority to other cell types has not been proven thus far. Furthermore, there has been some debate about the presence, function and importance of especially c-kit$^+$ cells, which invoked interesting discussions in the field.[45,46] The preclinical evidence for the added benefit of CSCs seems present and the first clinical trials (using c-kit$^+$ cells and cardiosphere-derived cells) have been completed, showing modestly positive results.[47,48]

Cardiomyocytes or cardiac progenitors, generated through either human embryonic stem cells (hESC) or induced pluripotent stem cell(iPSC) technology have recently also taken the stage as an alternative cellular therapeutic strategy. A first case report from France has already sparked enthusiasm towards clinical application of these cell types.[49] While no clinical study is under way at the moment, two non-human primate studies have shown both grafting and beneficial effects of hESC- and iPSC-derived cardiomyocytes.[50,51] The biggest concern from these studies is the non-lethal ventricular arrhythmias that occurred during follow-up, which will require further study before clinical application.[50,51]

Cardiac development: proliferation and differentiation

During embryonic development, nature is capable of forming a complete human being from a few omnipotent cells in the womb. The processes to form complete organisms are tightly regulated and are still being further elucidated for every organ in current research. These complex processes hold the secret of development and the reasons that any progenitor cell decides to turn into a specific differentiated cell type. Importantly, these phases include both cellular differentiation and proliferation in specific phases.

In embryonic development, the heart is the first organ to form. Through phase specific activation and inhibition of FGF(fibroblasts growth factor), Bmp(bone morphogenetic protein) and Wnt(wingless-related MMTV integration sites) signaling, mesodermal cells are committed to the cardiac lineage to form the cardiac crescents[52,53]. Cardiac progenitors are formed through a fetal gene program including Mesp1/2, Mef2c, Gata4, Nkx2.5, Islet1, Tbx5, and Hand2 which are gradually turned on to form the specific regions of the heart.[54,55] These specific regions include the generation and fusing of the first and second heart field (FHF and SHF) from the posteromedial and anterior mesoderm, which again have their own specific transcriptional signature.[55] After fusing to a single linear heart tube around embryonic day E8 and multiple looping and segregation steps from E8 to E14.5, the heart functions and expands its myocardial mass as a four-chambered muscle.[55]
Understanding and knowing these processes on a(n) (epi)genetic level allows us to explain certain congenital defects and also lets us study these processes *in vitro.*
Using hESC lines and the recently-discovered iPSC cells, we are able to study the developmental steps that a cell and organism undertake to ultimately become a cardiomyocyte, other cardiac cells and ultimately a fully functioning heart. Through specific reporter systems, we are able to select and study different populations of cardiac progenitor cells both *in vitro* and *ex vivo*, which are available in both the murine and human setting.[56,57] Certain pathways, like the Wnt signaling pathway, seem to affect these processes *in vitro* in pluripotent cells and also enhance direct cardiac reprogramming from fibroblasts.[58,59]
The cues for a (cardiac) cell to differentiate and especially proliferate are interesting from a therapeutic point of view, as the mature cardiomyocyte has a limited capacity to proliferate, especially at higher age.[17] In light of ischemic disease, one is stuck with what is left of your myocardium after a heart attack, with little endogenous capacity to regenerate. Trying to find new transcription factors that guide these crucial steps in development might reveal pathways that are necessary to restart these processes in a damaged heart. The re-entry in the cell cycle of endogenous cardiomyocytes has especially gained a lot of interest lately and might be amplified if one knows factors responsible for these processes in different phases.[18]

Thesis outline (see Figure 1)

Part 1: Looking back in cardiac development

In this thesis, we try to investigate the processes of cardiac progenitor proliferation and differentiation a little more. In **Chapter 2**, we review the Wnt-signaling pathway in cardiac development and disease, as this pathway has proven itself indispensable in important processes of cardiac cell proliferation and differentiation. In **Chapter 3**, we discuss an experimental setup, capable of screening for new compounds that induce cardiac progenitor proliferation, using *ex vivo* cardiac progenitors and compounds targeting the Wnt pathway as a positive control. In **Chapter 4**, we show an extensive qPCR-based transcription factor screen on different murine cardiac progenitor subtypes through the use of a double-reporter Nkx2.5-GFP/Islet1-dsRed murine ES-cell line, yielding multiple interesting transcription factors for further study. Hnf4α was the hit to subsequently investigate more closely, as it was not described in the heart before.

Part 2: Looking back in preclinical trials

We feel that much more can be learned from previously published research through systematic review and MA. By using both existing and novel techniques we were able to draw new conclusions on cell therapy research and large animal MI models that would have otherwise gone unnoticed. In **Chapter 5**, we report the effect of medication (regularly clinically prescribed after MI) on preclinical disease assays *in vitro* and *in vivo*, which likely have a great impact on clinical translatability of cell therapy and development of new therapeutics. In **Chapter 6**, we discuss our protocol for our systematic review and MA of preclinical studies using cardiac stem cells in MI, since it is important to make and show your research plan up front. In **Chapter 7**, the analyses described in Chapter 6 were executed, leading to the first systematic overview of cardiac stem cells in preclinical MI models, showing that multiple biases might have an impact on this research field and that large animal models differ significantly from small animal models with regards to efficacy and quality. In **Chapter 8**, we choose to extend these findings to other diseases. Using multiple previously published datasets, we show that the same heterogeneity might be present in the fields of neurological diseases and chronic kidney disease when applying cell therapy.

Since methodology needs to evolve together with research fields, we also tried to improve preclinical MA methodology. In **Chapter 9**, we used illustrative data simulations and empirical datasets, through which we were able to show that standardized mean differences in combination with its standard error results in false-positive publication bias assessments, which has currently gone unnoticed and has been erroneously applied by many fellow researchers. Finally, using a novel approach of multivariable meta-regression on control subjects' data in large animal MI models, we show in **Chapter 10**, that primary outcomes of large animal models are significantly dependent on multiple methodological factor like species, sex, MI model, comedication and follow-up time.

Part 3: Looking back in clinical trials

Not only preclinical trials can give us crucial extra information after their publication. Also, clinical trials datasets can help us if new questions need to be answered. In **Chapter 11**, we tested the effect of sham interventions by using MA and meta-regression on clinical control subjects. In **Chapter 12**, we reanalyzed the data of the REPAIR-AMI trial to characterize a new definition of responder identification after cell therapy.

Looking back to think ahead

The process of drug discovery and application testing to the clinical human setting is called translation. The core hypothesis of this thesis is that this translation is a two-way street. When moving forward towards clinical and preclinical scenarios for any drug-target in any disease, it is crucial to continuously go back to previous phases to answer and raise new questions, test new hypotheses and optimize a current therapy. We will move ahead most efficiently if we grant ourselves the time to look back too. In **Chapter 13** we combine all included chapters and put these in perspective to one another and current literature.

This thesis hopefully serves as an example of looking back in cardiac development and (pre)clinical studies, coming up with new hypotheses and crucial suggestions for model optimization and more efficient, translatable research in cardiovascular disease.

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2

Wnt/β-Catenin Signaling during Cardiac Development and Repair

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ABSTRACT

Active Wnt/β-catenin signaling is essential for proper cardiac specification, progenitor expansion and myocardial growth. During development, the mass of the embryonic heart increases multiple times to achieve the dimensions of adult ventricular chambers. Cell division in the embryonic heart is fairly present, whereas cell turnover in the adult myocardium is extremely low. Understanding of embryonic cardiomyocyte cell-replication, therefore, could improve strategies for cardiac regenerative therapeutics. Here, we review which role Wnt signaling plays in cardiac development and highlight a selection of attempts that have been made to modulate Wnt signaling after cardiac ischemic injury to improve cardiac function and reduce infarct size.

Introduction

Cardiovascular diseases and especially heart failure are among the most frequent disease entities worldwide. A loss of functional cardiomyocytes overtime can perturb the balance between the body's oxygen demands and the blood supply generated by the heart. Current therapies aim to prevent adverse cardiac remodeling, but do not restore the number of myocytes lost after myocardial infarction. Regeneration of myocardial tissue after myocardial infarction through endogenous renewal of cardiomyocytes is minimal.[1] Therefore, it is pivotal to understand the molecular and genetic factors that control cardiomyocyte proliferation and differentiation during early cardiac development, since it can provide crucial insights for cardiac regeneration. The heart is the first organ to be formed in the mammalian embryo, where its role becomes essential to supply the exponential increasing demands in nutrients as is required for growth.[2,3] The Wnt/β-catenin signaling pathway plays an important role in embryonic cardiac specification, cardiovascular progenitor expansion and cardiomyocyte proliferation.[4] Wnt signaling is rarely reported to be active in the adult heart; however, recent evidence suggests that after ischemic damage the myocardium and epicardium exhibit active Wnt signaling.[5,6] Here, we focus on Wnt signaling and its role in cardiac development. We also display a selection of efforts that have been made to modulate Wnt signaling after cardiac ischemic injury to improve infarct healing and functional outcome.

Wnt Signaling during Cardiogenesis

Cardiogenesis is a highly complex process that is governed by a dynamic interplay between embryonic growth pathways and transcriptional regulators controlling cell fate and specification.[3,7,8] Previous work from numerous laboratories has shown that the embryonic Wnt signaling pathway is essential during cardiogenesis and development.[9] Wnt signaling involves multiple complex signaling cascades, of which the β-catenin mediated canonical pathway and the non-canonical pathway are the most widely known.[10] The Wnt signaling system, consisting of 19 lipophilic proteins, controls wound repair and regeneration in simple organism such as planaria and hydra[11,12] to hair follicle, sweat gland and intestinal crypt regeneration in mammals.[13-15] Furthermore, Wnts are evolutionary conserved for their role in early development of the mammalian heart.[16-18] The heart in mammals is embryonically derived from the mesodermal layer, which rises from the primitive streak directly after gastrulation. Wnt signaling is required for the gastrulation process and in embryos from β-catenin knockout mouse the mesoderm failed to arise from the inner layer of endoderm cells.[19] During normal mouse cardiac development, the cells from the primitive streak migrate anteriorly to form the cardiac crescents of the splanchnic Mesp1/2+.[20,21] To achieve cardiac specification of these Mesp1/2+ cells Wnt/β-catenin is repressed in the cardiac mesoderm. The presence of a constitutive active β-catenin molecule in Mesp1+ cells abrogated cardiac tube formation in the mouse embryo, while antagonizing Wnt/β-catenin through Dkk-1 initiates cardiogenesis (Figure 1).[4,22,23] Around E7.5, the cardiac crescents arise from cardiac mesoderm, of which the posterior located crescent is characterized as the First-Heart-Field (FHF), whereas the anterior crescent is identified as the Second-Heart-Field (SHF). Tbx5 and Hcn4 were identified as markers of the early FHF and fate mapping experiments have shown that, in the posterior cardiac crescent, these Tbx5+ and Hcn4+ cells give rise to the left ventricle and parts of the atria.[24-27] Isl1 and FGF10 were found as specific markers of the SHF and lineage tracing revealed the contribution of Isl1+ cell populations to the right ventricle and

outflow tract on the arterial pole of the heart, whereas FGF10 also contributed to the venous pole of the early heart structure.[28-31] In mouse, the FHF and SHF regions fuse by E8 into a linear heart tube, followed by a looping process to eventually form the four-chambered heart.[2,32] From E8.5 on, bipotent Nkx2.5+ progenitors, contributing to the myocardial and smooth muscle lineages fuel an early increase in cardiac mass.[33] Isl1, a LIM homeodomain transcription factor, moreover, marks a distinct population of multipotent Isl1+ cardiovascular progenitors that play an essential role in sourcing the right ventricular myocardium and outflow tract.[28] Undifferentiated Isl1+ progenitors were shown to have the ability to give rise to endothelial cells, smooth muscle cells and cardiac myocytes.[34] Furthermore, it was shown that Wnt/β-catenin signaling controls the clonal expansion of Isl1+ progenitors. Mice with constitutively activated β-catenin in the Isl1 lineage showed to have a massive accumulation of Isl1+ cells in the SHF-derived structures as the right ventricle and the outflow tract, while conditional β-catenin loss of function studies revealed an arrest in development through attenuated expansion of Isl1+ progenitors (Figure 1).[18,35,36] After the specification of multipotent progenitors into committed ventricular progenitors or early cardiac myocytes, growth continues. And while the whole fetal heart is growing extensively, tight regulation per area is required. For many years, it is known that the outside layer, also called the compact myocardium, proliferates more rapidly when compared to the trabecular myocardium in luminal regions of the heart.[37,38] This region-specific proliferation of fetal cardiomyocytes is necessary for proper morphogenesis of ventricular myocardium, trabeculae and volume of chamber cavities. Recent work showed that Wnt/β-catenin regulates this regional expansion of ventricular myocytes (Figure 1). β-catenin was predominantly observed in the compact zone of the myocardium and when β-catenin was knocked out in ventricular cardiomyocytes this resulted in a reduction of the compact zone of the myocardium and an arrest in development around E12.5. Conversely, ubiquitous activated β-catenin in ventricular myocytes caused an increase in trabecular proliferation at E12.5. This increased ventricular proliferation was sustained until birth.[39,40]

To date, it remains unknown what exact role Wnt/β-catenin has in homeostasis of the adult myocardium. Moreover, it might play a role in endogenous cardiac repair and remodeling, since fetal gene programs, including Wnt signaling, are re-expressed upon myocardial infarction.[5,41]

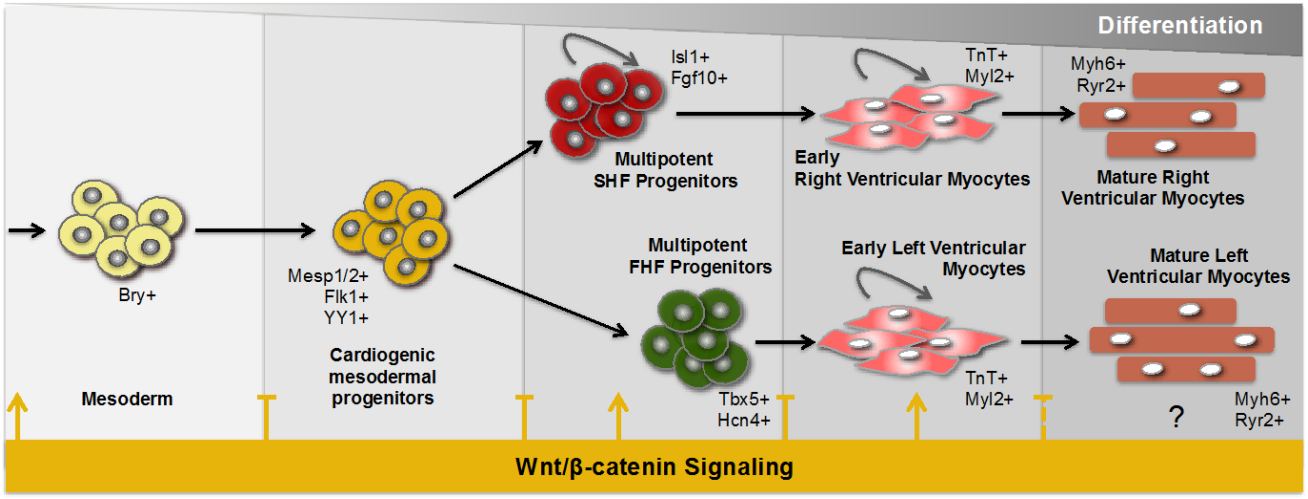

Figure 1. Distinct Phases of Wnt/β-catenin Signaling in Cardiac Development. Stage specific roles of Wnt/β-catenin signaling in proliferation and differentiation of cardiac progenitors and ventricular myocytes. Wnt/β-catenin is spatiotemporally activated or repressed to orchestrate normal cardiac formation; whereas activation of Wnt/β-catenin is required for mesodermal specification[42], repression of Wnt/β-catenin is mandatory for specification of cardiogenic mesodermal precursors and multipotent progenitors.[43] Subsequently, activated Wnt/β-catenin signaling has proliferative effects in multipotent progenitors[18] and early ventricular myocytes, while the repression of Wnt/β-catenin signaling in this stage promotes further differentiation and exiting of the cell cycle.[40] To date, it is unknown what exact effects Wnt/β-catenin exerts on adult cardiomyocytes. Arrows represent activated Wnt/β-catenin signaling; T's indicate repressed Wnt/β-catenin signaling; FHF, first-heart-field; SHF, second-heart-field.

Wnt Signaling during Cardiac Repair

Unlike neonatal heart tissue, the mammalian postnatal myocardium exhibits a very low turnover of cardiomyocytes or replenishment by resident progenitors, and therefore almost completely lacks the potential to regenerate.[1,44-46] Multiple tissues in the body with regenerative capacities, such as skin and liver, however, rely on embryonic growth pathways to compensate for lost cells or to refresh damaged tissue.[15,47] After ischemic cardiac injury the former functional adult myocardium is largely replaced by fibrotic scar tissue. Current therapies successfully minimize the duration of ischemia and pharmacologically prevent (adverse) remodeling of the remaining myocardium, but do not lead to restoration of the number of lost ventricular muscle cells.[48,49] At the cellular level, fetal gene programs are reactivated in response to myocardial damage.[41] Work from several groups has shown that modulation of canonical Wnt signaling in murine or rat cardiac ischemia models improved post-infarct outcomes, with various results (Table 1). Wnt signaling is activated when Wnt protein ligands occupy the Frizzled protein receptors.[50] In cooperation with lipoprotein receptor-related proteins, the Wnt signal is transferred over the membrane.[51] Subsequently, activated Dishevelled proteins disrupt the degradation-complex of glycogen synthase kinase 3 (GSK-3), APC and Axin, resulting in stabilization of cytoplasmic β-catenin and leading to nuclear accumulation.[52] Active nuclear β-catenin will activate the Wnt target genes with its cofactors LEF-1 and TCF.[53,54] Wnt signaling, therefore, can potentially be modulated at these described levels (Figure 2). In a permanent left anterior descending artery (LAD) ligation mouse model, Wnt signaling and its downstream effectors are activated upon infarction, manifested by increased levels of β-catenin, Dishevelled-1 and adenomatous polyposis coli (APC) protein in resident endothelial cells and newly formed vessels and an increase of Dishevelled-1 in the infarcted areas and border zones.[55,56]

Using an Axin2 promotor-driven LacZ-expressing murine myocardial infarction (MI) model, we

confirmed that post-MI several cell types react and express LacZ; 4 days after LAD ligation active Wnt/β-catenin signaling was observed in cardiomyocytes, smooth muscle cells, endothelial cells and progenitors.[5] Using a similar model with the TOPGAL (β-galactosidase gene driven by a T cell factor (TCF) β-catenin responsive promoter) mice, others subsequently showed that canonical

Wnt signaling is present 4 days post-MI in both subpericardial and perivascular endothelial cells and an increase in endothelial-to-mesenchymal transition, most likely upon Wnt activation.[57]

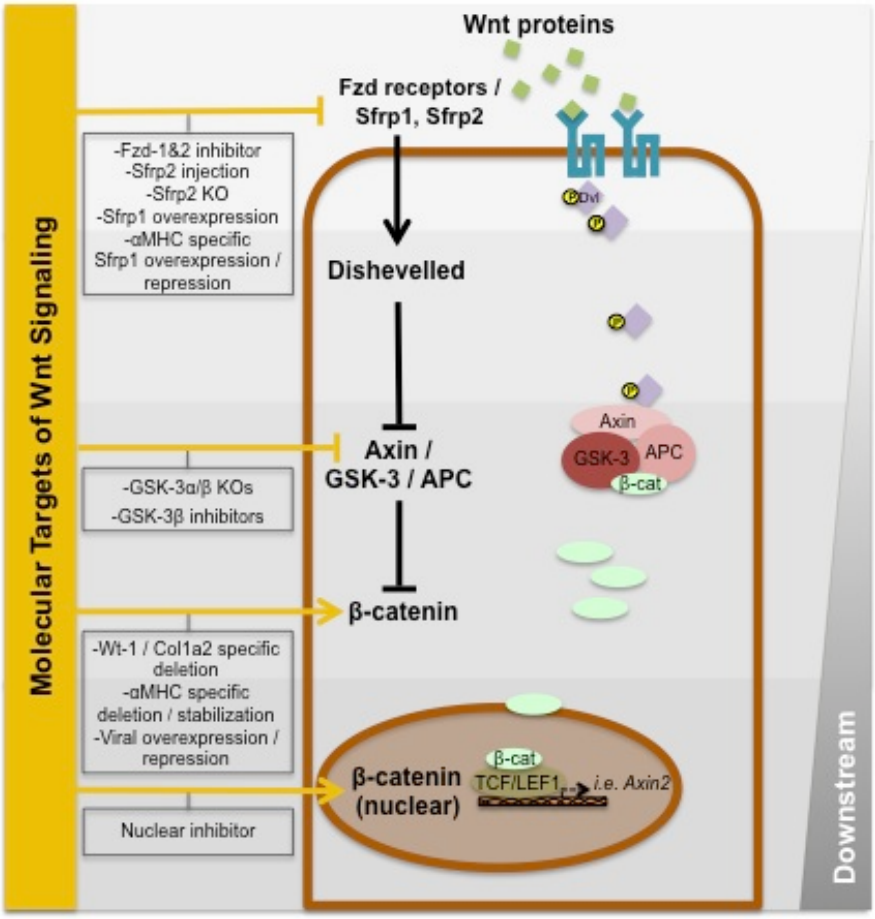

Figure 2. Selected Molecular Targets of Wnt Signaling Studied for Cardiac Regeneration. When Wnt proteins attach to the Frizzled-receptors, to activate Wnt/β-catenin signaling, Dishevelled is subsequently phosphorylated. Next, Dishevelled targets the Axin/GSK-3/APC destruction complex and thereby inhibits the degradation of β-catenin. As a result, β-catenin accumulates in the cytoplasm and will be translocalized to the nucleus where it binds to TCF and LEF-1 transcription factors to start transcription of direct downstream Wnt target genes as Axin2. Fzd, Frizzled; Sfrp, soluble frizzled related protein; GSK, Glycogen Synthase Kinase; KO, knockout; MHC, myosin heavy chain.

At the membrane level, overexpression of Secreted Frizzled Related Protein 1 (Sfrp1), which causes a decrease in Wnt signaling by occupying its target receptors as decoys, causes a reduction in infarct size and improved cardiac function.[58] The injection of recombinant Sfrp2 two days post-LAD ligation gave similar results.[59] Then again, genetic deletion of Sfrp2 showed reduced fibrosis and better cardiac function compared to littermate controls two weeks after LAD ligation.[60] Synthesized peptides, targeting the Frizzled-1 and Frizzled-2 receptor and thereby antagonizing Wnt signaling, also showed reduction of infarct area and increase in

repair.[61] Furthermore, alpha myosin-heavy-chain-driven overexpression of Sfrp1 caused decreased cardiac function and increase of the infarct size after ischemia-reperfusion and annihilated the effect that preconditioning has on reducing infarct size.[62] Presumably, timing and technical differences explain these controversies.

At the level of cytoplasmic degradation of β-catenin, inhibition of GSK-3, APC and Axin results in activation of Wnt signaling. Knocking-down both the α- and β-isoform of GSK-3 showed no difference in cardiac function after ischemic injury compared to wild type controls.[63] Knocking down GSK-3α caused an increase in mortality following LAD ligation, potentially via increased apoptosis and fibrosis and a decrease in cardiac function.[64] Targeting the other isoform GSK-3β, using either a genetic knock-out model or small inhibitory molecules (Lithium and SB216763), was associated with preserved cardiac function, less apoptosis and increased capillary density in the infarcted area.[65,66] However, it remains unknown if there is an actual refreshment of cardiomyocytes underlying this improved cardiac outcome. Furthermore, β-catenin itself, as a downstream target of GSK-3, APC and Axin, was targeted in several studies using different approaches. Vector based expression of β-catenin in mice caused better functional outcomes and decreased apoptosis compared to wild type controls.[67] Down-regulation of β-catenin in cardiac fibroblasts caused increased left ventricular dilatation *in vivo* and a decrease in fibroblast proliferation *in vitro* following ischemia.[6] Alpha myosin-heavy-chain (αMHC) specific deletion of β-catenin was superior over αMHC specific β-catenin stabilization, with significantly improved functional outcomes and an upregulation of cardiac fetal gene expression in animals lacking β-catenin in the heart.[68] A recent study used a murine LAD ligation model and treated mice immediately after ligation with an one-time injection of a Wnt inhibitor ICG-001, thereby antagonizing nuclear β-catenin; 10 days post-LAD occluded animals had better cardiac function and showed an increase in endothelial-to-mesenchymal transition compared with controls.[69]

These conflicting results may very well be due to the difference in timing, dosage and strategies. And while Wnt/β-catenin exerts highly stage specific effects during cardiac development, the same might be true for the process of ischemic cardiac damage and the endogenous repair mechanisms of the heart.

In conclusion, Wnt/β-catenin signaling plays a pivotal role in cardiovascular development. Moreover, Wnt signaling is evidently associated with the cellular mechanisms following cardiac ischemic injury. Thus far identified targets are Frizzled, GSK-3 and β-catenin. Current evidence, however, is lacking clear definite conclusions regarding cellular renewal of the myocardium, so further studies should aim to investigate the cell-specific stage-specific manipulation of Wnt/β-catenin signaling after cardiac ischemic injury and enhancement of endogenous repair.

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Conflicts of Interest

The authors declare no conflict of interest.

References	Target	Treatment/modulation	Wnt/β-cat	Timing	Outcome
[61]	Fzd-1/Fzd-2	Peptidergic Fzd-1/Fzd-2 antagonist UM206	↓	0 or 14d post-MI (similar results)	Reduction of infarct expansion and increased repair in infarct area
[59]	Sfrp2	Recombinant Sfrp2 injection	↓	Injection 2d post-MI	Reduced fibrosis and improved LVEF
[60]	Sfrp2	KO mice	↑	-	Reduced fibrosis and significantly higher LVEF compared to controls
[62]	Sfrp1	αMHC-specific Sfrp1 overexpression with doxycycline inducible repression	↓	Overexpression/repression (1wk prior to MI)	Ischemic preconditioning caused improved outcomes after MI due to GSK-3β inhibition, but this effect was diminished in Sfrp1-overexpressing CM
[58]	Sfrp1	Overexpression of Sfrp1	↓	-	Reduction of infarct size, fibrosis and improved cardiac function (7 d or 30 d post-MI)
[64]	GSK-3α	KO mice	↑	-	Increased mortality (10 d post-MI), more LV dilatation, dysfunction, hypertrophy, fibrosis and heart failure (8 weeks post-MI) and increased apoptosis in border zone (2 d post-MI) in KO mice
[66]	GSK-3β	KO mice (tamoxifen inducible)	↑	KO at 3d post-MI	Improved LVEF and LV dilatation with less hypertrophy post-MI in GSK-3β KO mice (8 weeks post-MI)
[63]	GSK-3	KO mice	↑	-	No functional difference between GSK-3 KO mice and controls
[65]	GSK-3β	Inhibitors (Lithium/SB216763)	↑	Directly after MI	GSK-3β inhibition mimicked ischemic precondition, resulting in less apoptotic cardiomyocytes and increased capillary density
[69]	β-catenin	Nuclear inhibitor (ICG-001)	↓	Directly after MI for 10d	Improved LVEF (10 d post-MI) and increased EMT in epicardial cells in treated mice
[6]	β-catenin	Downregulation (tamoxifen inducible) in cardiac fibroblasts	↓	10d prior to MI	Increased left ventricular dilatation (8 d post-MI) and decreased cardiac fibroblast proliferation *in vitro*
[68]	β-catenin	αMHC specific depletion or stabilization of β-catenin	↓/↑	-	Upregulation of fetal gene program (GATA4, Tbx5) and improved MI outcomes (LVEF and mortality) in β-catenin depleted animals compared to stabilization
[67]	β-catenin	Overexpression of β-catenin	↑	Directly after MI	Decreased left ventricular dilatation, increased fractional shortening and decreased apoptosis compared to controls (7 d post-MI)

Table 1. Selection of literature on Wnt modulation in cardiac ischemic injury. All studies used a left anterior descending artery (LAD) ligation model in mice or rats. An ischemia-reperfusion model was used in some studies ([62,66]). Fzd = Frizzled. Sfrp = soluble frizzled related protein. GSK = Glycogen Synthase Kinase. KO = knockout. MHC = myosin heavy chain. MI = myocardial infarction. LVEF = left ventricular ejection fraction. CM = cardiomyocytes. LV = left ventricle. EMT = endothelial-to-mesenchymal transition.

Author Contributions

Main text writing and figures: J.W. Buikema and P.P.M Zwetsloot; Editing and rewriting: P.A.F.M. Doevendans, I.J. Domian, J.P.G. Sluijter.

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3

Expanding Mouse Ventricular Cardiomyocytes through GSK-3 Inhibition

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ABSTRACT

Controlled proliferation of cardiac myocytes remains a major limitation in cell biology and one of the main underlying hurdles for true modern regenerative medicine. Here we provide a technique to robustly expand early fetal-derived mouse ventricular cardiomyocytes on a platform usable for high-throughput molecular screening, tissue engineering or potentially useful for *in vivo* translational experiments. This method provides a small molecule-based approach to control proliferation or differentiation of early beating cardiac myocytes through modulation of the Wnt/β-catenin signaling pathway. Moreover, isolation and expansion of fetal cardiomyocytes takes less than 3 weeks, yields a relatively pure (~70%) functional myogenic population and is highly reproducible.

Introduction

During cardiogenesis, cells from the endocardium, myocardium and epicardium give rise to the appearance of the ventricular wall.[1] The myogenic commitment of multipotent cardiovascular progenitors sources the myocardial compartment herein. And while the right ventricular myocardium originates from an Isl1/Nkx2.5 positive cell population, up to date, it remains unknown what transcription factor marks the origin of left ventricular myocardium. [2-4] Early Isl1+ and Nkx2.5+ right ventricular marked cardiomyocytes isolated from a double transgenic renewable cell source can be used for functional tissue engineering and show limited intrinsic capacity to proliferate *in vitro*.[5] Previous work showed that Wnt/β-catenin plays a pivotal role in self-renewal and myogenic differentiation of early embryonic multipotent progenitors.[6-8] In addition, recently, we demonstrated that Wnt/β-catenin signaling pathway also controls spatiotemporal proliferation and differentiation of early ventricular myocytes derived from pluripotent cell sources as well as mouse fetal ventricular myocytes. Furthermore, constitutively activated β-catenin in fetal ventricular myocardium promotes proliferation of cardiac myocytes in the left and right ventricle up to the early neonatal stage, while abrogation of β-catenin signaling attenuates proliferation of early ventricular myocytes.

Therefore, we explored the effect of a defined set of small molecules, known to modulate the Wnt/β-catenin signaling pathway, on proliferation and differentiation of early fetal-isolated ventricular myocytes. We found that a group of small molecules (Table 1), directly inhibiting cytoplasmic glycogen synthase kinase 3 (GSK-3) and thereby activating Wnt/β-catenin signaling, robustly enhanced the *ex vivo* proliferation capacity of early cardiomyocytes. Conversely, treatment with molecules abrogating Wnt/β-catenin signaling resulted in reduced intrinsic proliferation and enhanced differentiation as found with quantitative reverse transcription polymerase chain reaction (qRT-PCR) for structural cardiac genes.

Herein, we describe a reliable and reproducible method to isolate relatively high numbers of ventricular cardiomyocytes and provide the techniques to expand or differentiation these cells. Although, several strategies for isolation of rat and murine cardiac myocytes have been reported before, yet no easy and effective culture method exists to efficiently expand these. Mouse fetal (E11.5-14.5) cardiomyocytes have a limited capacity to proliferate ex vivo. This intrinsic capacity can be robustly enhanced with treatment of a GSK-3 inhibitor. Moreover, isolation and expansion or differentiation of fetal cardiomyocytes takes less than 3-weeks and yields high numbers of functional myocytes with relatively high purity (~70%).

Small molecule	Molecular target	Wnt/β-catenin signaling	Effect	Reference
BIO*	**GSK-3 inhibition**	**Activation**	**Proliferation**	**9**
CHIR99021	GSK-3 inhibition	Activation	Proliferation	10
1-Azakenpaullone	GSK-3 inhibition	Activation	Proliferation	11
IWR-1*	**Axin stabilization**	**Inhibition**	**Differentiation**	**12**
IWP-3	Porcupine inhibition	Inhibition	Differentiation	13
PNU7747	Nuclear β-catenin binding	Inhibition	Differentiation	14

Table 1. Selected small molecule inhibitors and activators of the Wnt/β-catenin signaling pathway.
GSK-3, glycogen synthase kinase 3. *Small-molecules used in this protocol.

BASIC PROTOCOL 1

Isolation of fetal ventricular cardiomyocytes

Introductory paragraph

This protocol is used to isolate ventricular cardiac myocytes from murine embryonic hearts (E11.5-14.5) using micro dissection and enzymatic digestion techniques. The steps describe how to dissect fetal tissues and how to process the cardiac tissue to yield dissociated cardiomyocytes. This protocol will yield between 50.000-200.000 ventricular myocytes per embryonic heart depending on the embryonic stage.

Materials

Sterile Phosphate Buffered Saline (PBS) 1x
Collagenase Solution (see Reagents & Solutions)
Trypsin/EDTA (Invitrogen, 5200-056)
Culture Media (see Reagents & Solutions)
15-ml centrifuge tubes (BD, 352097)
Scissor
Forceps
Scalpel
Microscope
Inverted light microscope
70% Ethanol
Laminar flow hood
37°C water bath
37°C / 5% CO_2 tissue incubator

Protocol steps

1. Euthanize mouse (preferable through cervical dislocation) and open up the abdominal skin and peritoneum with scissor and forceps.
2. Dissect out the uterus by cutting the following structures; Fallopian tube on one side, the cervix and the Fallopian tube on the other side. Transfer the uterus containing the embryos to a 10cm cell culture dish with ice-cold PBS 1x (Figure 1A).
3. Open up the uterus wall with a scissor and collect the embryos by opening up the yolk sacs. Cut-off the head with one incision in the neck and the lower structures with one incision above the liver. The heart now becomes visible and can be dissected-out. Remove the pericardial sac, atrial and vascular tissue (Figure 1B).
4. Incise ventricles multiple (3-4) times with a scalpel and put in 15mL collection tube(s) while in PBS 1x on ice (Figure 1C)
5. Wash ventricles with PBS 1x, spin down at 850 RPM for 3 minutes and discard wash buffer. Repeat this step up to 3 times.
6. Add 2-4mL of Collagenase Digestion Solution to 15mL tube and incubate ventricular tissue for 1-1.5 hours at 37°C water bath.

7. Gently pipet ventricular tissue up and down every 15 minutes to enhance enzymatic digestion.
8. After 1-1.5 hours, add 1 volume of 0.25% trypsin and incubate for 3 minutes at 37°C.
9. Gently pipet up and down to create a single cells suspension and neutralize the trypsin with 1 volume of FBS.
10. Fill up 15mL tube(s) with PBS1x and spin cells down for 5 minutes at 850 RPM.
11. Discard supernatant and re-suspend cells in 1mL of Culture Media (see Reagents & Solutions).
12. Pipet up 10µL and count cells using standard counting method.

Step annotations

1. Note: Experiments involving live animals must be according to the institutional regulations and require approval of the Institutional Animal Care and Use Committee (IACUC) or equivalent.
2. Note: The mouse uterus is located in the peritoneal cavity. The uterus tube runs from the cervix up to the Fallopian tubes on both sides.

4. Optional: The left and right ventricle can be collected separate if the study design requires this.

6. Note: Alternatively, the 15mL can be placed horizontally on rotational platform in a 37°C incubator to enhance enzymatic digestion with continuous mechanical force.

9. TROUBLESHOOTING: Before pipetting the tissue up and down in the digestion buffer, wet pipet tips to avoid adhesion of cardiac tissue on the inside wall.
10. IMPORTANT: Pipet gently to avoid shear stress.

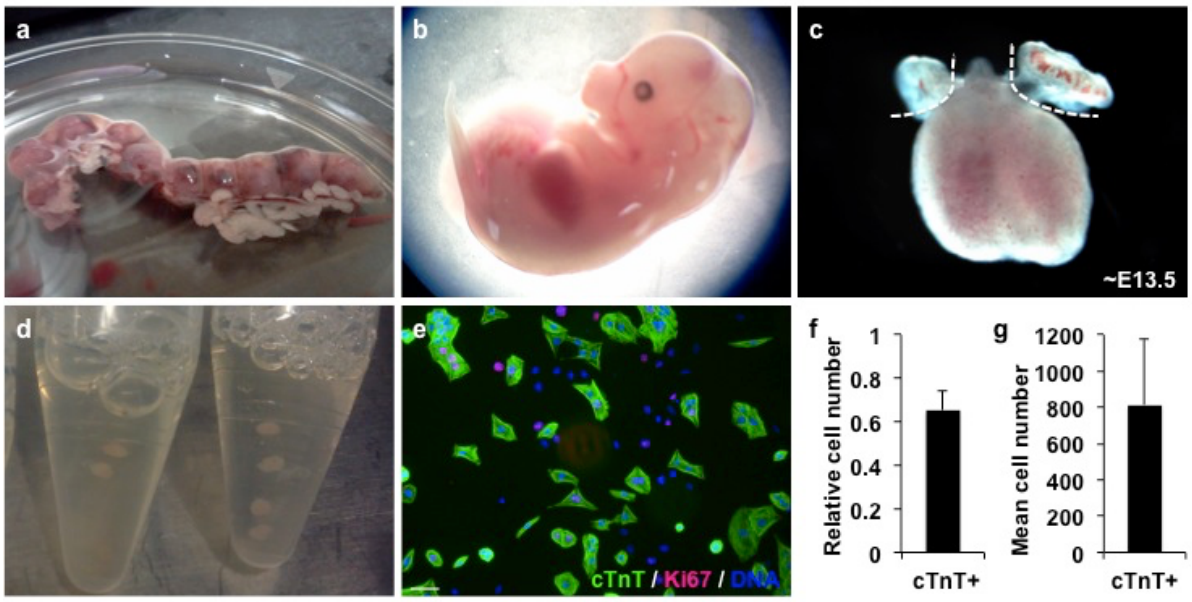

Figure 1 Isolation and plating of ventricular myocytes. (a) Image of dissected mouse uterus containing multiple embryos. (b) mouse embryo at ~E12.5. Dashed lines indicate where incisions should be made to yield the heart. (c) fetal mouse heart. Dashed lines indicate excision of atrial tissue. (d) pooling of ventricular tissue in 15mL tubes. 2-3 tubes can be used for biological replicates. (e) representative image of ventricular cells stained for cardiac troponin T (cTnT) (green), Ki67 (red) and DAPI (blue). (f) percentage of cTnT+ cells 1 day after isolation (E12.5+1). Scale bar represents 50µm. (g) quantification of cTnT+ cell number per well of a 384-well plate. Error bars indicate standard deviation. (n=3, each in 6 technical replicates).

BASIC PROTOCOL 2

2-Dimensional culture of fetal ventricular cardiomyocytes

This protocol describes the procedures for the in vitro culture of fetal-derived ventricular cardiomyocytes. Depending on the purpose of the experiment, cells can be plated (2-dimensional) or cultured in aggregates (3-dimensional) (**alternate protocol 2**) (Table 2). 2-dimensional cultures allow better quantification options for proliferation or differentiation assays, while 3-dimensional cultures preserve a better physiological cellular context useful for tissue engineering experiments. GSK-3 inhibitor treatment in a 2-dimensional culture conditions results in up to a 20-fold increase of cardiac myocyte within one week (Figure 2A-D).

Materials

0.1% Gelatin solution (see Reagents & Solutions)
Collagen solution 1:20 (see Reagents & Solutions)
Culture Media (see Reagents & Solutions)
6-Bromoindirubin-3'-oxime (BIO) (Sigma, B1686) (see Reagents & Solutions)
Inhibitor of Wnt Response-1 (IWR) (Sigma, I0161) (see Reagents & Solutions)
Sterile pipet basin
Multichannel pipet
24, 96 or 384-well cell culture plate(s)
Inverted light microscope
Laminar flow hood
37°C water bath
37°C / 5% CO_2 tissue incubator
4% Paraformaldehyde (PFA) solution
TRIzol Reagent (Invitrogen, 15596-026)

Protocol steps

1. Coat plates with 0.1% gelatin solution for 20 minutes at RT.
2. Re-suspend cells in the desired amount of culture media to end-up with cell densities per volume as listed in table 2. Next, transfer the cell suspension to a sterile pipet basin.
3. Use a single or multichannel pipet to plate cells on 0.1% gelatin pre-coated plates.
4. Allow the cells to settle overnight in a tissue incubator at 37°C (12-24 hours)
5. The next day, dissolve compounds in DMSO to create a 10mM stock concentration.
6. Prepare 10x of final concentrations in Culture Media (for BIO make 15-25µM and IWR 80-160µM solutions (which is 10x if the appropriate end concentration, i.e. 1.5-2.5µM for BIO and 8-16µM for IWR)
7. Add the small-molecules and DMSO carrier control in 10x concentration of final to the cell culture (i.e. add 8.3µl of 10x compound to 75µl of media in a 384-well, to make ~83µl)
8. Change the Culture Media every 2-3 days, or when color changes to orange/yellow, and add compounds again in 10x of the final concentration on top.
9. Fix cells with 4% PFA at desired time-point(s) and perform standard immunocytochemistry. Alternatively lyse unfixed cells in TrIzol Reagent and process for RT-PCR analysis.

Step annotations

1. NOTE: To mimic a more organic environment plates can also be coated with collagen type I solution for 20 minutes at RT.

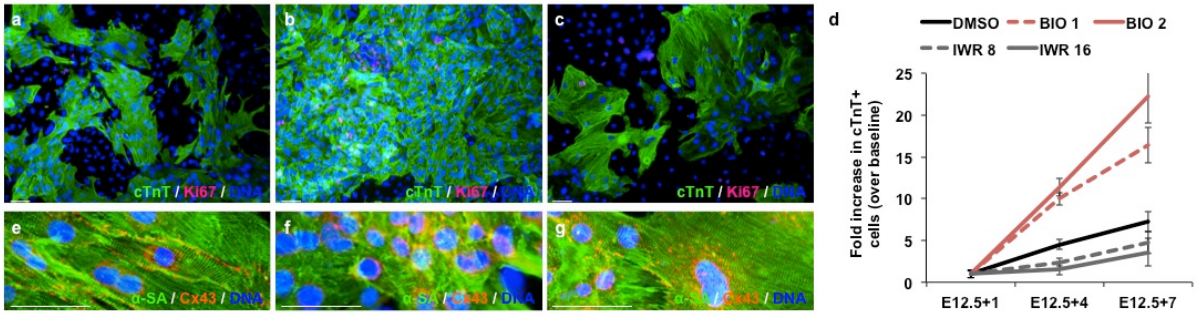

Figure 2. 2-dimensional expansion of differentiation of ventricular myocytes. Representative images of ventricular cells cultured in (a) DMSO, (b) BIO or (c) IWR stained for cardiac troponin T (cTnT) (green), Ki67 (red) and DAPI (blue). Scale bar represents 50μm. (d) Quantification of cTnT+ cells at day 1 (baseline) (E12.5+1), 3 (E12.5+4) and 6 (E12.5+7) additional days of culture in the presence or absence of BIO or IWR. Error bars indicate standard deviation. (n=3, each in 6 technical replicates for each time point).

ALTERNATE PROTOCOL 2

3-Dimensional culture of fetal ventricular cardiomyocytes

3-Dimensional aggregate culture of cardiomyocytes has the advantage that physiological cell-cell communication is maintained or re-established to some extent. Because of the absence of attachment to the culture plate, cardiomyocytes form aggregates which allow interaction and mechanical forces in 3-dimensions. Previous work suggested that neonatal rat ventricular cells have an innate potential to re-form the complex 3-dimensional organization of cardiac tissue *in vitro*. The electrophysiological properties of cardiomyocytes cultured in 3-dimensional environment were superior to those of the same cells cultured as monolayers [15,16]. Using **alternative protocol 2**, ventricular cells can be cultured, expanded or differentiated using a hanging drop aggregate method.

Materials

Culture Media
TRIzol® reagent (Invitrogen, 15596-026)
Chloroform (Fisher Scientific, 67-66-3)
Pipet basin
Multichannel pipet
Inverted light microscope
Laminar flow hood
37°C water bath
37°C / 5% CO_2 tissue incubator

Protocol steps

1. Re-suspend cells in a density of 25-50 cells/µl and add final concentrations of small molecules (BIO 1.5-2.5µM and IWR 8.0-16µM)
2. Use a multichannel pipet to make 10µl drops (250-500 cells/drop) on a petri dish.
3. Flip the petri dish upside down and place hanging drops in tissue culture incubator at 37°C.
4. Within 12 hours the isolated cardiac myocytes will cluster and form cardiac microspheres. Within 24-36 hours spontaneously contracting cardiac microspheres can be observed.
5. After 2-4 days, pool cardiac microspheres into petri dish or low-attachment with new culture media for additional culture or process tissue for optical, immunohistochemistry or standard real-time reverse-transcription PCR analysis (RT-PCR).

IMPORTANT NOTE: For optimal cell adhesion, it is recommended not to move the plate within the first 12 hours of cardiac microsphere formation.

SUPPORT PROTOCOL 2

Analysis of expanded or differentiated fetal ventricular myocytes

This section summarizes and discusses a selection of analytical readout options available to study proliferation and/or differentiation of fetal-derived ventricular progenitors.

Materials

PBS 1x
Saponin (Sigma, 47036)
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) 1:10.000 (invitrogen, D1306)
Primary antibodies
Cardiac Troponin T (cTnT) 1:250 (mouse monoclonal, NeoMarkers, Ms-295)
Ki67 1:300 (rabbit monoclonal, Abcam 16667)
α-Sarcomeric Actinin (α-SA) 1:250 (mouse monoclonal, Sigma, A7811)
Connexin-43 (Cx43) 1:150 (rabbit polyclonal, Sigma, C6219)
Secondary antibodies
Alexa 488nm donkey anti-mouse 1:400 (Invitrogen, A-21202)
Alexa 594nm donkey anti-rabbit 1:400 (Invitrogen, A-21206)
Immunofluorescence microscope
Qiagen RNeasy Mini Kit (Qiagen, 74104)
iScript cDNA Synthesis Kit (BIO-RAD, 170-8891)

Immunofluorescence

Standard immunofluorescence can be used to visualize cell number, proliferation and differentiation or commitment. Commonly used antibodies for a basic analysis of cardiac myocytes (and/or differentiation or commitment) are cTnT and α-SA. Assays to analyze cell number and proliferation include staining for nuclear DNA with DAPI or for proliferation marker Ki67. In this protocol, cTnT staining is used to discriminate between the cardiomyocyte and non-cardiomyocyte populations. Furthermore, scoring of total cTnT+ cell number, as well all Ki67+/cTnT+ cells are

useful methods to evaluate proliferation.

Quantitative PCR
RT-PCR analysis for structural cardiac genes provides insights in what effect of small-molecules exert on gene expression. In this protocol, it is illustrated for cardiac Troponin T (TnnT2), ventricular specific myosin light chain (Myl2), and cardiac specific α-myosin heavy chain (Myh6) in ventricular myocytes treated with BIO or IWR. Furthermore, activation or inhibition of a molecular pathway can be monitored by RT-PCR analysis for direct target genes.

REAGENTS AND SOLUTIONS

Collagenase solution
Phosphate Buffered Saline (PBS) 1x, Collagenase A (Roche, Cat. No. 11 088 785 103) 1mg/mL, Collagenase B (Roche, Cat. No. 11 088 823 103) 1mg/mL and 20% Fetal Bovine Serum (FBS) (Gemini
Bioproducts)

Trypsin
0.25% Trypsin-EDTA 1x (Invitrogen, 25200-056)

0.1% Gelatin solution
Phosphate Buffered Saline (PBS) 1x, 0.1% gelatin (Sigma, G1890)

Collagen 1:20 solution
Phosphate Buffered Saline (PBS) 1x, collagen type I 3.37mg/mL 1:20 (BD, 354236)

Ascorbic Acid
Sterile water, Ascorbic acid 10mg/mL (100x) (Sigma, A4544)

Culture media
Iscove's Modified Dulbeccos Medium (IMDM) (Thermo Scientific, SH30228.01), 10% Fetal Bovine Serum (FBS) (Gemini Bioproducts, 100-500), Non-Essential Amino Acids solution (NEAA) 1x (Invitrogen, 11140-050), Ascorbic acid 1x, Pencillin 50U/mL, Streptomycin 50µg/mL, Mercaptoethanol 1:150.000 (Sigma, M6250)

BIO (GSK-3 inhibitor)
6-Bromoindirubin-3'-oxime (BIO) (Sigma, B1686), prepare 10mM solution in DMSO

IWR (Axin inhibitor)
Inhibitor of Wnt Response-1 (IWR) (Sigma, I0161), prepare 10mM solution in DMSO

Antibodies
Primary antibodies: Cardiac Troponin T (cTnT) 1:250 (mouse monoclonal, NeoMarkers), Ki67 1:300 (rabbit monoclonal, Abcam 16667), α-Sarcomeric Actinin (α-SA) 1:250 (mouse monoclonal, Sigma, A7811), Connexin-43 (Cx43) 1:150 (rabbit polyclonal, Sigma, C6219). Secondary antibodies: Donkey anti-mouse Alexa 488nm 1:400 (Invitrogen, A-21202) and donkey anti-rabbit 594nm 1:400 (Invitrogen, A-21206).

COMMENTARY

Background Information
Annual ventricular myocyte turnover is estimated around 2% in the adult mammalian heart and occurs mostly through refreshment of preexisting myocytes.[17] Unlike the mammalian heart, certain fish and amphibians maintain the capacity to repair cardiac damage throughout life.[18] And while adult mammalian myocardium is almost completely lacking the capacity to regenerate the myocytes lost after injury, it was shown that the early neonatal myocardium has an intrinsic capacity to reconstitute for myocyte loss. This capacity of neonatal cardiomyocytes to proliferate is lost early after birth. The neonatal intrinsic cardiomyocyte response to reconstitute the cell loss, however, is similar to the regenerative capacity of zebrafish hearts.[19,20]
Pharmacological or cell based therapy, aiming at replacing or augmenting the number of functional myocardial cells represents an attractive therapeutic approach to regenerate the injured mammalian heart. These pharmacological compounds or cells will have to be applied or assembled into the 3-dimensional structure of the myocardial wall. Boosted cardiomyocyte renewal or direct engrafted cardiac cells will then have to be functionally coupled with native myocardium to improve cardiac function. Furthermore, electrophysiological coupling of *de novo* cardiomyocytes has to occur without resulting in arrhythmias or rejection. For such a pharmacological or cell-based approach to regenerate the adult heart, a more detailed understanding of physiological cardiac myocyte growth and turnover is required.
Up to date, no stable cardiac myocyte cell-line has been described. And although neonatal rat cardiomyocytes have a limited capacity to proliferate *ex vivo*, neonatal mouse-derived myocytes almost completely lack the intrinsic capacity to further proliferate. Recent work, however, showed that a number of microRNAs efficiently promote the proliferation of murine cardiomyocytes.[21] In this regard, having a small-molecular strategy to direct early cardiomyocytes to expand or further differentiate forms therefore the next step to cardiomyocyte culture. Furthermore, the setup of this protocol allows it to study molecular Wnt signals driving the proliferation and differentiation. In addition, this approach is adaptable into a platform to identify novel small-molecules regulating early cardiomyocyte fate.

CRITICAL PARAMETERS AND TROUBLESHOOTING

Survival and viability
Low yield is often a result of too much shear stress through vigorously pipetting or too long

exposure to enzymatic digestion. Since the cardiac cells in the native myocardium are highly organized and tightly connected to each other by gap junctions and adherens junctions (desmosomes) it requires slow enzymatic dissociation over 1-2 hours. In addition, gentle pipetting enhances the dissociation process and shortens the digestion time. Therefore, the survival and viability of the isolated cells is a balance between the least shear stress and the shortest possible digestion process. To optimize cell dissociation, a 3-minute Trypsin digestion step can be added after 1-2 hours of collagenase treatment. Optionally collagenase digestion can be performed on a rotational shaker.

Adherence

Protein coating of the cell culture plates is necessary to facilitate sufficient attachment of plated cells. As described, we routinely use gelatin and collagen protein-solutions for coating of our culture plates. In addition, fibronectin and laminin are other proteins often used for coating. If adherence of cell is an issue, protein concentrations in the coating solution can be increased up to a 10-fold to promote cell adhesion.

Plating density

For successful expansion of ventricular myocytes, it is important to start off with the seeding densities as described in Table 2. For RT-PCR analysis it is recommended to use higher densities, while for cell count analysis lower cell numbers per well are time saving.

Environment (Plate)	Media (volume)	Cells (number)
2-Dimensional culture		
24-well culture plate	1000µl	15.000-45.000
96-well culture plate	200µl	2.500-7.500
384-well culture plate	75µl	500-1500
3-Dimensional culture		
10 or 15 cm petri-dish	10-20µl/drop	250-500

Table 2. Overview of 2 and 3-dimensional culture methods

Small-molecule treatment

It is important to add compounds within 12-24 hours after cell seeding to maintain ventricular myocytes in a proliferative state, while it is not recommended to seed cells together with the final concentration of the compounds, since compounds can have different effects on cell survival, viability and attachment. Furthermore, avoid multiple freezing and thawing cycles of the small molecule stocks and instead prepare small aliquots. 10x diluted compounds are usually last for up to 1 week. Cover tubes with small-molecules in aluminum foil to avoid light exposure.

Contamination

Under aseptic conditions ventricular myocytes cultures can be grown without antibiotics. However, we prefer to perform the animal dissection and isolation of cardiac cells under non-sterile conditions and supplement the Culture Media with low concentrations of antibiotics (see Reagents

& Solutions: Culture Media). Potentially, antibiotics can cause bacterial resistance and can interfere with the expansion or differentiation of cardiac cells.

Anticipated Results

This protocol describes in detail the isolation and subsequently expansion or differentiation of fetal ventricular cardiomyocytes. As described in **Basic Protocol 1**, between 500.000 and 2 million ventricular myocytes can be isolated from one litter, depending on the embryonic stage. Usually, between 60-80% of the fetal ventricular myocytes stain positive for cardiac troponin T (cTnT) at day 1 (E12.5+1) (Figure 1E-G).

As shown in **Basic Protocol 2**, isolated cells can subsequently be plated and up to a 20-fold expanded in 1 week with GSK-3 inhibitor treatment. Conversely, inhibition of Wnt signaling with IWR, results in the reduced proliferation and ventricular myocyte cell number (Figure 2A-D). Furthermore, expanded and differentiated ventricular myocytes show strong sarcomere expression and gap junction formation (as illustrated in Figure 2E-G).

Alternate Protocol 2, describes the culture of ventricular myocytes in aggregates. When BIO or IWR are added to the aggregates, it results in increased or decreased diameter of the cardiac tissue aggregates compared to the DMSO control (Figure 3A-D). Furthermore, IWR appears to increase the beating rate while BIO has the opposite effect, when compared to aggregates cultured in DMSO (Supplementary Video 1-3). Quantitative RT-PCR reveals that BIO decreases and IWR increases mRNA expression of structural cardiac / ventricular genes as Tnnt2, Myl2 and Myh6 as compared to the DMSO controls (Figure 3E).

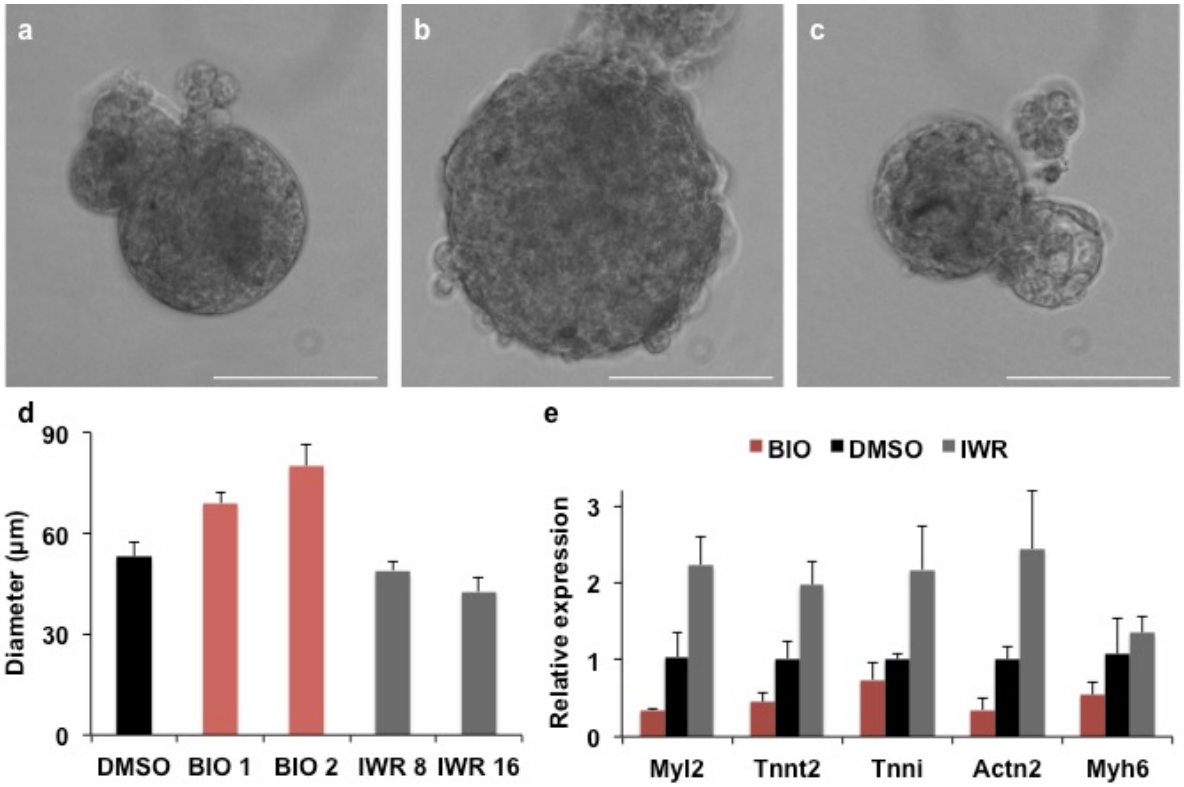

Figure 3. 3-dimensional culture of ventricular myocytes. Representative bright field images of ventricular cells cultured in aggregates treated with (a) DMSO, (b) BIO or (c) IWR. Scale bar represents 50µm. (d) Quantification of the diameter of ventricular tissue constructs in µm. (n=3, each in 3 technical replicates). (e) qPCR analysis for structural cardiac genes on cells treated with BIO, DMSO or IWR. (n=3). Error bars indicate standard deviation.

TIME CONSIDERATIONS

Animal breeding

The animal breeding for this protocol exists of setting up C57BL/6 or CD1 females with males. Mice mate during night, so plug checks should preferably be performed in the in the morning. Usually, C57BL/6 or CD1 females plug within 3 days in the presence of a male. Fetal cardiomyocytes can be yielded between E11.5 and 14.5. Total time for this part of the protocol is 2-2.5 weeks.

Isolation

Sacrificing the pregnant female and dissecting the fetal ventricular tissue takes approximately 1-1.5 hours. Digestion of tissue 1-2 hours and plating varies on the size of the experiment. Total time is approximately 2-4 hours.

Expansion

Expansion or differentiation assays can be performed varying from 3 days up to 1-2 weeks. Total time of this part depends on experimental design.

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4

Hnf4α plays a role in the embryonic heart, but seems dispensable in adulthood

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In preparation

ABSTRACT

The formation of the embryonic heart is governed by multiple transcription factors, which are all spatiotemporal expressed. The identification of lowly expressed transcription factors, guiding these tight processes is crucial for our understanding of cardiac formation and cardiomyocyte proliferation and differentiation. Here we report the discovery of a new first heart field-specific transcription factor, Hnf4α, identified in a high-throughput quantitative PCR based transcription factor array. Small molecule inhibitors of Hnf4α promote expansion of embryonic stem cell-derived first heart field progenitors. In the adult heart, Hnf4α could be detected on a protein level, but was hardly detectable on corresponding transcriptional levels. This makes it less likely that Hnf4α has a prominent role in the adult heart. The role of Hnf4α in the developing heart remains to be elucidated.

Introduction

Cardiac formation is orchestrated by spatiotemporal and structural expression of many regulators. Transcription factors (TFs) play a central role in cardiogenesis by functioning as activators or repressors of multiple genes.[1-3] TFs are proteins that bind to a specific DNA sequence, thereby controlling the genetic transcription from DNA to mRNA. TFs are essential for the regulation of gene expression and are evolutionary conserved in all living organisms. The mouse genome encodes for approximately 2000 TFs, and whereas some TFs are highly tissue specific, others are involved in the gene regulation of multiple tissues.

Genes are often flanked by several binding-sites for distinct TFs. This allows for an organ-unique fingerprint regulated by network of TFs acting on tissue specific genes.[4-6] Previous work has shown that Mef2, Nkx2.5, Tbx5 and Gata4 are key transcription factors with high expression levels necessary for cardiac specification and development.[3,7-9] The reprogramming of somatic cells into cardiomyocytes requires overexpression of Gata4, Mef2 ,Tbx5, Hand2 and underscores their pivotal role in cardiogenesis.[10,11] However, TFs with a low expression level are usually not identified in standard whole genome gene expression studies or array approaches, because they are less likely to be picked up, relative to background signals.[12] To be able to identify these lowly expressed TF involved in these processes, a method called Quanttrx has emerged, using quantitative PCR.[13] Identifying lowly expressed TFs, involved in early cardiomyogenesis might reveal pathways involved in cardiac differentiation and proliferation, which could pave the way for both better biological understanding of cardiomyogenesis, understanding of cardiac genetic defects and new potential therapeutic targets for induction of cardiac regeneration. As recent evidence confirms the proliferation of endogenous cardiomyocytes during adulthood, understanding the cues for turning on these programs is highly relevant for cardiac regenerative strategies[14,15], as both myocardial damage and chronic hypoxia also seem to trigger a slumbering mechanism of cardiac proliferation.[15,16]

The early mammalian heart arises from two regions of multipotent progenitor cells in the splanchnic mesoderm, described as the first (FHF) and second-heart-field (SHF) located posteriorly and medially to the cardiac crescent, and anteriorly to the pharyngeal mesoderm.[17] The FHF progenitors of the cardiac crescent coalesce along the midline and give rise to the primitive linear heart tube and ultimately the majority of the cells of the left ventricle and inflow tract.[18,19] Cells from the SHF contribute to the growth of the developing heart and eventually give rise to the right ventricle, outflow tract and parts of the inflow tract.[20,21] Fate mapping experiments have revealed specific molecular markers, such as Isl1 for the SHF[22,23], Tbx5 and Hcn4 for the FHF[18,24,25] and WT-1 and Tbx18 for the proepicardium.[26,27] Recent work has shown the isolation of distinct FHF and SHF transcriptional color-marked progenitors derived from embryonic stem (ES) cells.[28] This system can be used to further explore regional expression of transcription factors and specific transcriptional cues for proliferation and further differentiation.

Here, we report the identification of Hepatocyte Nuclear Factor 4 Alpha (Hnf4α) in ventricular regions of the developing heart, specifically in FHF cardiac progenitors. While present in the developmental stage, we could not confirm Hnf4α presence and relevance in the adult heart.

Methods

Mouse ES cell culture and differentiation

ES cell culture protocol was adapted from a previously published protocol.[29] Murine Nkx2.5-eGFP and AHF-Mef2C-DsRed double transgenic embryonic stem (ES) cells[28] were maintained in regular serum containing media (DMEM, 15% FCS, pen/step 1x, NEAA 1x, L-Glutamine, LIF and 2-Mercaptoethanol (2-ME)) in an irradiated MEF feeder system. Prior to differentiation, cells were adapted for 2 days on 0.1% gelatin-coated polystyrene plates in (IMDM, 15% FCS, pen/strep, NEAA, L-Glutamine, LIF and 2-ME). At day 0, cells were dissociated with 0.25% trypsin for 3 minutes and re-suspended in a density of 100.000 cells/mL. Differentiation was induced by embryoid bodies (EBs) in hanging drops of 1000 cells in 10µl of differentiation media (IMDM, 15% FCS, pen/strep, NEAA, L-Glutamine, Ascorbic acid 50ng/mL and 2-ME). At day 3 of differentiation EBs were pooled in a ratio of 4:1.

FACS isolation of cardiac-tagged ES-derived cardiac progenitor populations.

E9.5 embryos and D6 EBs were trypsinized into single cell suspension to FACS isolate GFP+/DsRed- (FHF), GFP+/DsRed+ (SHF), GFP-/DsRed+ (Mef2c) or GFP-/DsRed- (NEG) cell populations. After isolation, populations were spun down and lysed in Tryzol (Qiagen) or plated on gelatin-coated polystyrene plates.

Quanttrx

Quanttrx was performed on four ES cell-derived cardiac progenitor populations. Isolated RNA was linearly amplified to desired quantities. cDNA was generated and high-throughput quantitative PCR was performed in 384-well plates for ~1850 transcription factors of the mouse genome. All data was normalized to the NEG populations. Pierson correlation of data was performed with the Genepattern software from the Broad Institute. Heatmaps were generated with GenePattern HeatMapViewer software from the Broad Institute.[30]

RNA isolation and quantitative PCR of ES-derived FACS-sorted cardiac progenitors

RNA was extracted and purified with RNeasy Mini Kit (Qiagen). Using the iScript cDNA synthesis kit (BioRad). cDNA was generated and quantitative PCR was performed with HOT-START SYBR Green (USB/AffyMetrix) on an Eppendorf Mastercycler for 40 cycles. For shown data, analysis was performed on threshold cycles lower than 36. Primer sequences are available on request.

Immunohistochemistry ES-derived FACS-sorted cardiac progenitors

Cells were cultured in 384- or 96-well plates. Cells were blocked for 2 hours at room temperature in 5% FCS of secondary antibody and 0.1% Saponin in PBS. Overnight incubation at 4º C was performed for primary antibodies in the presence of 2% FCS of secondary antibody and 0.1% PBS. Following primary antibodies were used: cardiac Troponin T (mouse monoclonal, NeoMarkers, 1:200), Ki67 (rabbit monoclonal, Abcam, 1:300). Secondary staining was performed with Alexa fluor 488nm-, 594nm-conjugated antibodies (Invitrogen, 1:400) against the appropriate species for ~2 hours at room temperature. Finally, nuclei of cells were visualized with ProLong Gold Antifade Reagent with DAPI blue (Invitrogen) before fixation with 4% performaldehyde.

Imaging of ES-derived FACS-sorted cardiac progenitors

Images and movies were taken with a Leica DMI4000B immunofluorescence microscope connected to Leica Application Suite Advanced Fluorescence 3.0.0 software package with similar exposure times for all samples within experiments. Brightness of images was edited with Adobe Photoshop respecting the same increase for all images shown within one experiment.

Organs and embryos for immunohistochemistry and protein/RNA measurements

Hearts and livers of C57BL/6-mice were used for protein and RNA measurements. All organs were homogenized using a Bead Beater (Precellys 24, Bertin Technologies). Embryos were taken from sacrificed pregnant C57BL/6-mice, retrieved on E12.5. Organs and embryos used for slides were paraffin-embedded directly after dissection.

Neonatal rat cardiomyocytes (NRCs)

Neonatal rat pups of the strain RccHan:WIST were terminated by decapitation. Hearts were excised aseptically and vessels were removed. NRCs were isolated by washing the biopsy in SolA (80g NaCl, 4g KCl, 10g glucose, 0.6g $Na_2HPO_4.2H_2O$, 0.6g KH_2PO_4, 0.2g phenol red, 47,7g HEPES in 1L MilliQ and sterilized by filter for 10x stock) and by mincing the heart in ±2mm^3 pieces. The fragments were further dissociated using a solution comprised of 2,5% trypsin, 4mg/mL DNAse in 1x SolA. After digestion, the solution was centrifuged and resuspended in HAM's F10 (ThermoFisher, #11550043) supplemented with 5% FCS and 1% L-glut. Cells were added to an uncoated 20cm^2 polystyrene dish for 2 hours, after which non-adherent cells were collected, centrifuged and resuspended in HAM's F10 and counted. 100.000 cells were plated overnight(o/n) on laminin coated coverslips.

Hl-1 cell line

Hl-1 cells were cultured as previously described[31] in Claycomb media (Sigma, #51800C), supplemented with 10% FCS, 2mM L-glut, 0.1mM norepinephrine and 1% penicillin/streptomycin. Cells were lysed in Roche Lysis buffer for western blots.

CGR8 ES-derived cardiomyocytes

Murine CGR8 ESC were used for generation of ES-derived cardiomyocytes. A serum-free differentiation method was used, as described previously.[32] Briefly, through an EB-method, cells were stimulated with Activin A and BMP4, after which the Wnt-antagonist IWR1 was introduced to induce efficient cardiac differentiation.[32] Cells were plated on coverslips for subsequent imaging.

Western blots

Samples were lysed in Roche cOmplete Lysis Buffer (Roche, #0471996400). 10µg of protein was loaded on iBlot™ gels, which were ran for ±1hour at 165V. Transfer was done using the iBlot™ system (according to manufacturer's protocol), after which the membrane was blocked with 5% BSA and incubated o/n with 1:1000 primary antibody in 5% BSA at 4 degrees. The next day the membrane was incubated with secondary HRPO-labeled secondary antibody, (1:1000 in 5% BSA, targeting the species of the primary antibody) for 1 hour. Membranes were analysed using

chemiluminescence substrate (Sigma CPS1120-1KT) and imaged using a BioRad ChemiDoc^{tm} MP imaging system and ImageLab 5.1 software.
Primary antibodies used were goat anti-HNF4α (Santa Cruz, #C-19) and rabbit anti-GAPDH (Cell Signalling, 2118S). Secondary antibodies were rabbit anti-goat (DAKO, #P0449) and goat anti-rabbit (Sigma, #P0448).

Immunohistochemistry of organs, embryos and NRCs

Slides were deparaffinized through multiple Xylene and graded alcohol washing steps. Antigen retrieval was performed, using 10mM citrate buffer (pH 6.0) in boiling water for 30 minutes. Quenching was performed in 3% H_2O_2. After 2 hours of blocking in 1% BSA, slides were incubated with 1:100 primary antibody in blocking solution (anti-Hnf4α (Cell Signaling, C3313) and anti-α-actinin (Sigma)) o/n at 4 ^{0}C. The next day, slides were washed with PBS three times, and incubated with secondary proteins (1:400 in blocking solution, as previously described for the ES-derived FACS sorted cells) for 2 hours at room temperature. After washing two times, DAPI (1:10000 in PBS) was applied for 15 minutes, and the last washing step was performed. Slides were mounted with Vectashield (Vectorlabs, #H-1000), after which coverslips were applied.
For NRCs, immunohistochemistry was performed, after 4% PFA fixation for 20 minutes. The same staining protocol as for ES-derived FACS-sorted cardiac progenitors was applied. Antibodies used were anti-Hnf4α (Cell Signaling, C3313) and anti-α-actinin (Sigma, C1A4).
All images of slides were taken with an Olympus BX53 immunofluorescence microscope, again using similar exposure times for all samples within experiments.

qPCR on organs

RNA isolated from TryZol samples was treated with TURBO DNAse (Ambion, AM2238) and converted to cDNA using iScript cDNA synthesis kit (Bio-Rad 170-8842). qPCR was ran, using SYBR-Green (BioRad, #1708880) on a Thermal Cycler (Bio-Rad, C-1000). Primer sequences are available upon requests.

Sequencing

PCR products were purified by adding 0.5 µL exonuclease I (20U/µL, NE Biolabs, #MO293S) and 1.0 µL Shrimp Alkaline Phosphatase (1U/µL, Westburg, # 2660A) to each sample. Big Dye terminator (BDT) reaction V1.1 (Life technologies, #4337452) was done, after which thermocycling was performed. Retrieved products were ran on a DNA Analyzer (Thermo Fisher, #3730).

SiRNA experiments

NRCs were cultured for 6 hours in OptiMem (ThermoFisher, #31985062) containing siRNAs and lipofectamine RNAiMax in a ratio 1:1 (final concentration 5pmol siRNA/well). anti-HNF4α siRNA (Invitrogen, MSS205166 cat# 1320001, stealth siRNA 20nmole, species mouse) and scrambled siRNA (Ambion, Cat#4390827 Custom select siRNA5nmole) were used. After 6 hours of transfection, medium was changed to normal HAM's F10 medium and cells were cultured for another 66 hours, after which cells were fixed using 4% PFA. Importantly, the selected siRNA was selected for its predicted targeting of all known isoforms in multiple species.

Statistical Analysis

Statistical analysis was performed with a one-way ANOVA. P-values <0.05 were considered statistically significant.

Results

A recently developed method for the quantitative analysis of transcriptional components (Quanttrx) allowed us to study the transcriptional control in cardiac progenitor progenitors from the FHF and the SHF.[12,13] We induced cardiac differentiation in a previously reported ES cell system, carrying a double fluorescent cardiac reporter system.[28] From day 6 (D6) differentiated embryoid bodies, we FACS-isolated four distinct populations: an AHF-Mef2c+/Nkx2.5- (Mef2c), AHF-Mef2c-/Nkx2.5+ (FHF), AHF-Mef2c+/Nkx2.5+ (SHF) and AHF-Mef2c+/Nkx2.5- (NEG) genetically marked cell population. After linear amplification of all samples, we performed high-throughput Quanttrx TF analysis (Figure 1A). In an averaged Pierson correlation, we identified a set of genes expressed in specifically the FHF progenitors known for liver development and maintenance (Figure 1A). Hepatocyte Nuclear Factor 1 Alpha and Beta (Hnf1α and Hnf1β), Hepatocyte Growth Factor Receptor (Met) and especially Hnf4α, were upregulated in FHF progenitors, but not in SHF marked cells (Figure 1A).

Hnf4α has been characterized as a master regulator TF, playing a pivotal role in liver development and homeostasis in mice and humans.[33,34] We therefore focused on Hnf4α and validated expression levels in ES-derived and embryo-derived transgenic marked Mef2c, SHF, FHF and NEG cell populations (Figure 1B). We found that Hnf4α mRNA expression was relatively enriched in FHF marked cells at D6 of *in vitro* differentiation (Figure 1B) and E9.5 of *in vivo* development (Figure 1C). To evaluate the expression pattern in time, we isolated FHF marked cells at D6 and plated these on gelatin-coated surfaces for 3 days. We did qPCR analysis for Hnf4α at D6 and each day thereafter (D6+1, +2, +3). In culture, we found that Hnf4α levels decreased rapidly within 2-3 days after isolation from embryoid bodies ($p<0.05$) (Figure 1C). We found similar results in E11.5 hearts. Hnf4α expression in cardiac tissue at this stage was neglectable when compared to E9.5 heart and E11.5 fetal liver ($p<0.001$) (Figure 1D). Hnf4α seems to be transiently expressed in FHF cardiac progenitors. At a transcriptional level, we gathered evidence that Hnf4α is spatiotemporal expressed in FHF-derived progenitors or a subset of this lineage.

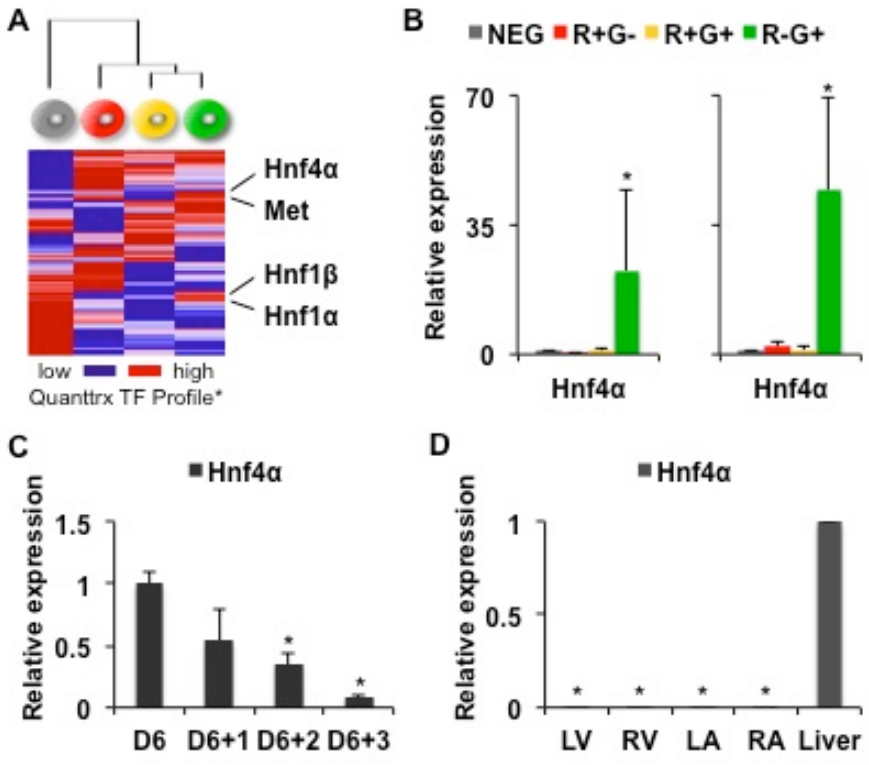

Figure 1. High-throughput quantitative PCR identifies Hnf4α in the First-Heart-Field. (A) Pierson clustering of Quanttrx transcriptional factor analysis in ES cell-derived AHF-Mef2c+/Nkx2.5- (R+G-), AHF-Mef2c-/Nkx2.5+ (R-G+), AHF-Mef2c+/Nkx2.5+ (R+G+), AHF-Mef2c+/Nkx2.5- (NEG) genetically marked cell populations. (B) Quantitative PCR validation for Hnf4α in day 6 (D6) ES cell-derived progenitors (left graph) and E9.5 embryo-isolated progenitors (right graph). (C) Quantitative PCR analysis for Hnf4α in ES cell-derived R-G+ cells at D6, and after plating for 1, 2 or 3 days (D6+1, +2, +3). (D) Hnf4α gene expression in E11.5 hearts and livers at different regions; left ventricle (LV), right ventricle (RV), left atrium (LA) and right atrium (RA). Error bars indicate standard deviation. N=3 biological experiments.

Hnf4α is a nuclear receptor protein, mostly expressed in adult liver, gut, kidney and pancreatic β-cells. Hnf4α was originally classified as an orphan receptor protein continuously influencing fatty acid metabolism and bound to a number of fatty acids. The endogenous ligand is linoleic acid, however its role remains unclear.[35] Additionally, recently a group of small molecules was identified that exert antagonistic effects on Hnf4α.[36] We therefore treated sorted FHF progenitors with these Hnf4α inhibitors named BI6015 (BI6) and BIM5058 (BIM). After 6 days of additional culture we stained for the cardiac marker Troponin T and proliferation marker Ki67. As shown in Figure 2A-D we found a ~4 fold increase in Troponin T positive cells when treated with BIM or BI6 compared to the DMSO control ($p<0.001$ for all compared to DMSO, BIO was used as a positive control). Remarkably, the proliferating cells were predominantly observed in isolated clusters, suggesting proliferation rather than differentiation of these cardiac progenitor cells.

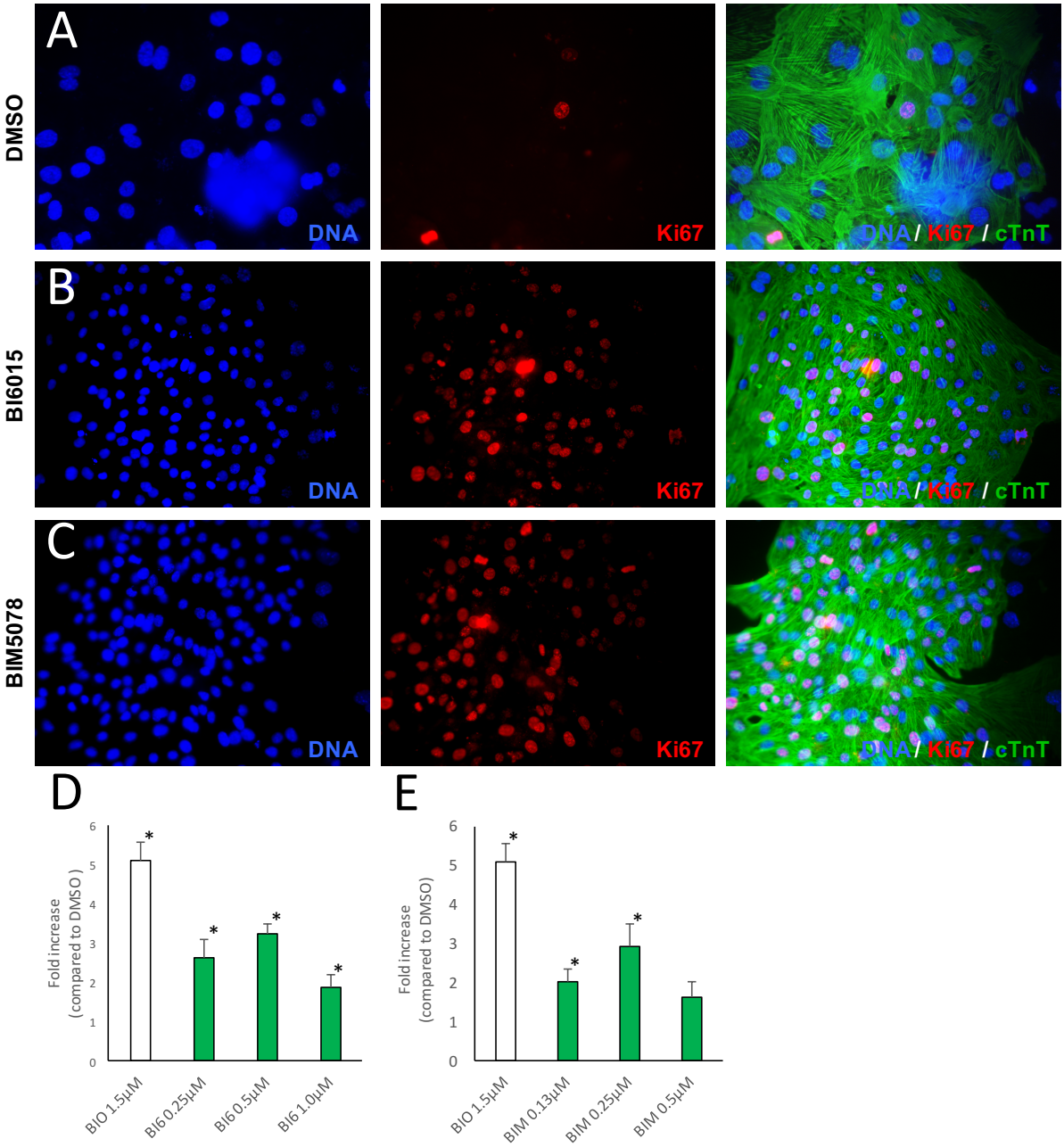

Figure 2. Hnf4α antagonists promote proliferation of First-Heart-Field derived progenitors. Immunohistochemistry for cardiac Troponin T (cTnT) (green), Ki67 (red) and Dapi (DNA) (blue) in cells treated with (A) the carrier control or (B) Hnf4α antagonist BI6015 or (C) Hnf4α antagonist BIM5073. (D) Cell count of cTnT+ cells treated with Hnf4α inhibitor BI6015 compared to DMSO. (E) Fold increase of cTnT+ cells treated with Hnf4α inhibitor BIM5073 compared to DMSO. Error bars indicate standard deviation. N=3 biological experiments for all samples.
*p-value<0.01

Next, we wondered if Hnf4α was also present in the adult heart. We performed Western blots on multiple cardiac samples, confirming Hnf4α presence in the mouse heart (Figure 3A), neonatal rat cardiomyocytes (Figure 3D) and a murine Hl-1 cell line (Figure 3F), using liver samples as a positive control. We also checked the presence in both healthy and diseases human heart samples (Figure 3D, hypertrophic, dilated and ischemic cardiomyopathies), showing presence, but no difference in terms of Hnf4α protein expression. Importantly, protein presence was confirmed using multiple Hnf4α-antibodies on Western blots (data not shown).
We also specifically immune-stained for Hnf4α, showing an unexpected presence in the intercalated disc in both murine hearts (Figure 3B) and on the border of connecting neonatal rat

cardiomyocytes (Figure 3E), while our embryonic liver control showed only nuclear expression (Figure 3C). We also confirmed this specificity in murine ES-derived cardiomyocytes *in vitro*, again localizing to the borders of the cells (Figure 3H). Next, we wondered if we could change Hnf4α expression and potential downstream effects, using SiRNAs, hypothetically targeting Hnf4α in all species. However, Hnf4α protein expression on staining was not altered after SiRNA treatment of 3 days (Figure 3I-J).

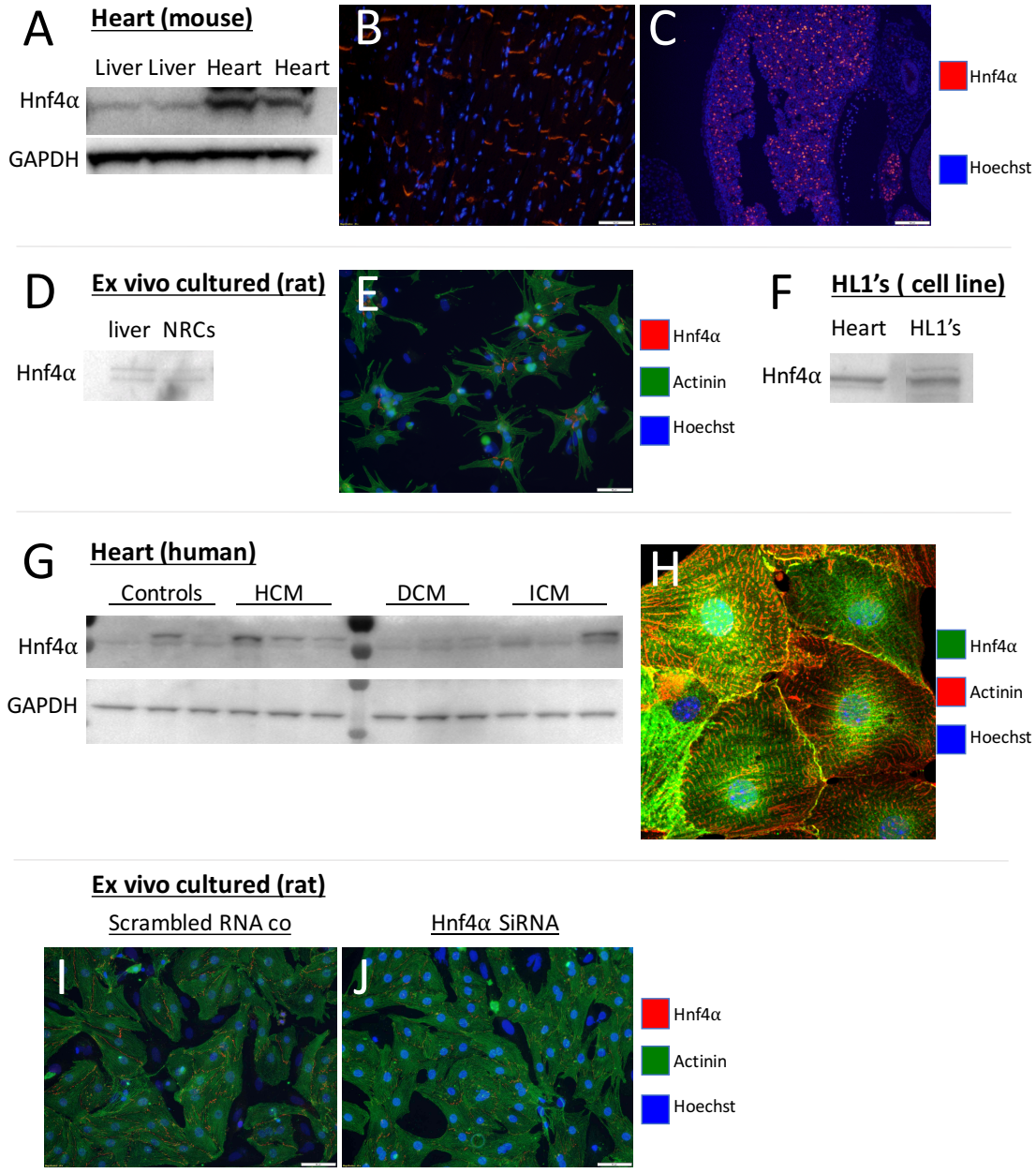

Figure 3. Hnf4α seems present on a protein level. (A) Western blot for murine lysed hearts and livers show protein expression in both organs. (B) Staining for Hnf4α reveals specific localization to the intercalated disc in the murine heart (C) Staining for Hnf4α shows known nuclear localization in embryonic liver samples (D) Western blot for lysed neonatal rat cardiomyocytes shows Hnf4α protein expression (E) Staining for Hnf4α shows localization on borders of neonatal rat cardiomyocytes *in vitro* (F) Western blot for lysed Hl-1 cells *in vitro* suggests presence of Hnf4α protein(G) Western blot suggests presence in human cardiac samples of Hnf4α protein, with no obvious differences between diseases (H) Staining for Hnf4α shows localization to borders of ES-derived cardiomyocytes *in vitro* (I-J) SiRNA treatment in neonatal rat cardiomyocytes does not result in reduced Hnf4α protein expression in rat neonatal cardiomyocytes.

Subsequently, we also performed qPCR on adult cardiac tissue, to confirm the presence of Hnf4α in the adult heart on an RNA-level. While our liver control gave normal-range positive results by qPCR, the adult heart did not show the amount of expression we expected based on the protein assays and liver controls, showing a 10-cycle difference, compared to the liver for two different primer pairs (Figure 4A). Running the qPCR product on a gel also showed a different size for one primer pair's PCR-product, while the other one showed a similar size, albeit lower expressed, product compared to its liver control (Figure 4B). We subsequently sequenced these products, resulting in multiple potential candidates, among which Hnf4α. However, also products like ion channels were among the proposed transcripts with similar coverage of base pairs.

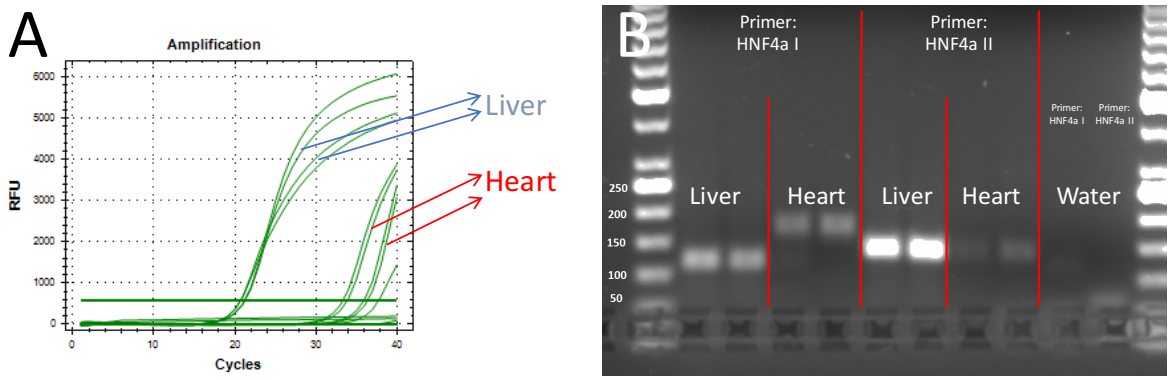

Figure 4. Hnf4α cannot be confirmed on an RNA level. (A) qPCR cycles show a discrepancy when loading the same RNA content. (B) run on a gel, qPCR products are less and in the case of Primer I also of different size, compared to liver controls.

Discussion

The cardiac regulatory network of TFs, which tightly controls growth and specification of region-specific progenitors to secure morphogenesis and organ size, has been relatively uncovered.[37,38] Our data shows that Hnf4α, which is known to be important for liver, pancreas and gut development and homeostasis[39-41], also plays a role in early cardiac development. *In vitro* inhibition of Hnf4α results in increased proliferation of a subset of ES cell-derived FHF progenitors, raising the possibility that Hnf4α regulates growth and specification of early cardiac cells in specifically the FHF. The absence of Hnf4α on a transcript level in the adult phase, makes it unlikely that Hnf4α is highly present in the adult heart, although expression in a small subpopulation cannot be excluded. Rather, it might be an Hnf4α-like protein that resides in the adult intercalated disc. As the antibodies used for staining, target the ligand-binding domain of Hnf4α, it might be possible that another protein with a fatty-acid-binding domain resides in the intercalated disc, which is both an intriguing and novel hypothesis to pursue. This hypothesis would also translate to the human situation, as similar patterns are also observed in human cardiac tissue, as can be seen on www.proteinatlas.org.[42]

Except for key transcription regulators, most TFs are relatively low expressed. This causes problems when conventional chip-based genome expression arrays are used to identify region specific TFs. For this work, we used a published method based on high-throughput qPCR

analysis[12,13], which led to the identification of a set of liver TFs in ES cell-derived FHF cardiac progenitors. In the meantime, new methods like RNA-sequencing have emerged that have replaced Quanttrx-like methods in the last years. Two recent papers have used single-cell RNA sequencing to look for the spatial expression patterns of the developing heart in multiple regions.[43,44] These groups looked in E8.5-E10.5[43] and E9.5 till postnatal day 21[44], respectively, identifying new cardiac region-specific expression patterns for the developing murine heart. Hnf4α was not among the identified differently expressed genes, although the focus was mostly on large changes in RNA expression. Furthermore, these time-points might already be too late to observe the same Hnf4α expression we saw in our experiments, as we also saw a steep decrease over time. Earlier time-points might be more appropriate in our case. Interestingly, there is already proof for the influence of Hnf4α in early cardiac development through endodermal Hnf4α-signaling, influencing early mesoderm.[32] In this paper, Hnf4α-siRNAs also reduced early cardiac differentiation directly, supporting our hypothesis.[32] If this phenomenon is bimodal, coming both from endodermal and mesodermal Hnf4α-expression, remains to be studied. Interestingly, recent work discusses the importance of Hnf4α in early cardiac development; pregnant mice fed the Hnf4α-inhibitor showed same RNA expression patterns in their progeny as 'congenital heart defect'-causing agents like trichloroethylene.[45]

Future work should aim to more precisely characterize the embryonic presence and location of Hnf4α in time. Work from several groups shows a relation between Hnf4α and Gata4 and Gata6 expression in the liver. Gata4 plays an essential role in cardiogenesis and Gata4 knockouts are arrested in development between E7.0 and E9.5. Mutant embryos lack a primitive heart tube because the two bilaterally symmetric promyocardial primordia fail to migrate ventrally, and instead, result in two lateral independent heart-like tubes.[9,46] Therefore, it would be of great interest to show the interaction of Hnf4α and Gata4 in cardiac cells. The role of Hnf4α has been studied in multiple organs. Conditional deletion of Hnf4α in the liver results in liver growth and dysfunction.[40] In the gut, Hnf4α represses Wnt signaling in crypt cells, and genetic deletion results in over-proliferation of crypt cells.[41] Furthermore, Hnf4α binds to the predicted consensus sites of Lef1[47], which is one of the downstream effectors of Wnt signaling, which might result in increased β-catenin signaling, resulting in dedifferentiation and proliferation of hepatic cells.[48] Our work in vitro, suggest a similar effect in FHF cardiac progenitors, potentially inhibiting any differentiation signaling through Wnt signaling, like shown previously by our own group.[49] As both Hnf4α and Wnt signaling are also involved in processes like fatty acid metabolism[50], this might explain the presence of Hnf4α and the increased proliferation of cardiac progenitors in this study when Hnf4α is inhibited. However, a cardiac specific deletion of Hnf4α is mandatory to fully elucidate its role in regulation of the FHF and cardiac development in general.

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5

Can new therapeutics beat current MI medication in cardiovascular disease assays?

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In preparation

ABSTRACT

Background

Many interventions have led to a reduction in mortality and morbidity in cardiovascular diseases, including the administration of multiple drugs after myocardial infarction(MI). These drugs all have their impact on processes during cardiovascular disease, yet are not controlled for in our experiments.

Methods

We used meta-analysis to compare preclinical studies for cell therapy omitting or using comedication throughout the study. We subsequently went back to the lab and tested the effect of clinically prescribed MI medication (aspirin, ticagrelor, metoprolol, captopril and atorvastatin) on *in vitro* human cardiovascular disease assays of angiogenesis, fibrosis, immune response and apoptosis. Also, the additive effect of cell therapy through human mesenchymal stem cells (hMSCs) was tested.

Results

Meta-analysis revealed a significant decrease in efficacy for cell therapy over placebo for preclinical studies using comedication. Angiogenesis assays and myofibroblast differentiation experiments showed a positive influence of MI medication and cell therapy *in vitro*. Immune response through immunoglobulin excretion assays and released factors by hMSCs in culture did not seem to be affected by comedication.

Conclusion

We observed effects of clinically prescribed MI medication on some cardiovascular disease assays, which is not being corrected for in current preclinical research. This might explain a part of current translational failure of new cardiovascular therapeutics, including cell therapy. Clinically prescribed medication should be added to confirmatory *in vitro* and *in vivo* therapeutic studies to optimize translational success.

Keywords: myocardial infarction; clinically prescribed medication; translational failure; cell therapy

Abbreviations

MI	myocardial infarction
hMSC	human mesenchymal stem cell
HUVEC	human umbilical vein endothelial cell
hCF	human cardiac fibroblast
hiPSC	human induced pluripotent stem cell
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
hPBMC	human peripheral blood mononuclear cell

Introduction

The past decades have given us many new therapeutic strategies after myocardial infarction (MI), all causing marked reductions in adverse cardiac events and mortality.[1] The introduction of percutaneous interventions and multiple drugs (aspirin, P2Y12-inhibitors, β-blockers, ACE/Angiotensin-inhibitors and statins) have all made substantial impact on cardiovascular disease burden.[1] However, we are still in search of additional therapies, as cardiovascular disease and its growing chronic nature, remain an issue. Especially the minimization of cardiac damage during/after injury and the incapability of repairing the heart after damage are main focuses nowadays in biomedical research.[2] For this, multiple therapies are being studied, like anti-inflammatory compounds and cellular therapeutics, which both have reached clinical phases with mixed results.[2] It seems we are less able to translate potential promising therapies to actual clinical interventions that show an additional benefit on top of standard care, sometimes referred to as 'translational failure'.[3]

The process of therapeutic research usually starts in the lab and involves many stages, going from *in vitro* to *in vivo* studies (going from small to large animals) in exploratory and confirmatory phases, before starting clinical trials. *In vitro*, multiple processes of the disease are studied; e.g. angiogenesis, fibrosis, immune responses and (reduction of) cell death. Interestingly, many of the clinically prescribed MI drugs are described to affect these processes and primary outcome measurements.[4-9] This is crucial, as these drugs potentially influence the same assays and mechanisms that serve as readouts and checkpoints in our current biomedical research for new therapeutics. Furthermore, these drugs might even interact with one another, blunting previously positive results from a single compound.[10] Pharmaceutical companies do extensive interaction studies on new compounds to make sure that new drugs do not act on existing drugs directly.[11] In contrast, we barely make use of the addition of drugs in our actual *in vitro* and *in vivo* disease models, despite their presence in the clinical situation of the disease under study and their known effects on disease progression and beneficial effects on the processes. These drugs are for the first time regularly added in clinical phases of research, based on our current guidelines of standard clinical care.

Our hypothesis is, that the (non-)administration of regularly prescribed MI drugs might explain part of the reduction in efficacy when translating any new therapy from the lab and preclinical models to clinical situations. This is especially relevant in situations where mechanisms of the therapy are only partially known and multimodal, like cell therapy. The mechanisms of cell therapy are thought to be mainly paracrine (especially in the case of human mesenchymal stem cells(hMSCs))[12], thereby potentially competing for the activation of the same processes as clinically prescribed drugs. Furthermore, biological therapeutics like cell therapy might be positively or negatively influenced by the presence of these five compounds.

Through meta-analysis of large animal studies and subsequent testing of the additive effect of clinically prescribed drugs on commonly used human *in vitro* assays for multiple processes (angiogenesis, fibrosis, immune response and excreted factors), we show in this study that regular comedication might alter the efficacy of cell therapy and affects some of the cardiovascular processes we study, creating reduced (and more realistic) therapeutic windows for any new therapy under study.

Methods

Meta-analysis dataset

We used a previously published dataset of placebo-controlled large animal MI studies investigating the efficacy of cell therapy.[13] Outcomes used were ejection fraction, end systolic volume and end diastolic volume. For both volumes, a standardized mean difference was used, as volumes cannot be directly compared across different species. In addition to the published data, we recorded the use of any of clinically prescribed MI drug classes (aspirin, P2Y12-inhibitors, β-blockers, ACE/Angiotensin-inhibitors and statins) throughout the whole study in both therapy and placebo group. A study using one or more of these was regarded as using 'comedication', while no use meant 'no comedication'. Studies only administering the drugs during induction of the MI were not considered as adequately administering these drugs.

Clinically prescribed MI drugs

The compounds used in our *in vitro* experiments were aspirin (Selleckchem #S3017), ticagrelor (Selleckchem #S4079), metoprolol (AstraZeneca, 1mg/ml), captopril (Selleckchem #S2051) and atorvastatin (Selleckchem #S2077). The combination of all 5 will be referred to as 'comedication'. All compounds were selected for their common use and being an active metabolite in dilution. Metoprolol was dissolved in sodium chloride (3.6mg/ml) with a stock concentration of 3mM, the other compounds were dissolved in DMSO in a 10mM stock concentration. All DMSO-dissolved drugs were stored at -80 ^{0}C. metoprolol was stored at -20 ^{0}C. All drugs where dissolved in corresponding culture media at the start of the experiment. As a control, similar amounts of DMSO were used.

hMSCs

Fetal human hMSCs (donor UMCU281109) were used as the additive biological therapeutic. hMSCs were maintained in MEM-α (Gibco #22561), supplemented with 10% fetal bovine serum (FBS, Gibco #10270(Lot #41G2740K), 100U/ml penicillin and 100 μg/ml Streptomycin (P/S) (Lonza #17-602E), 1ng/ml bFGF (Sigma #F0291) and L-ascorbic acid-2-phosphate (Sigma #A4034). hMSCs were maintained at 5% CO_2, 20% O_2 and 37 C° and maintained as published previously.[14] Passages 8-15 where used for experiments. For conditioned media, hMSCs were plated for at least 48 hours, after which the media was collected and immediately used in the appropriate assay. For the exosome-secretion assay, exosome-free media was used (regular media, spun down at 100,000g for 30 minutes). The human adipokine array kit (R&D Systems, #ARY024) was used to measure excreted growth factors. hMSCs were cultured in the same media, but with 0.5% FBS instead of 10% for 72hours, after which the supernatant was centrifuged (1000rpm) and used for further analysis according to the manufacturer's protocol. Array was analyzed using Image J and the Protein Array analyzer plug-in.[15]

Human Umbilical Vein Endothelial Cell (HUVEC) tube formation assay

To test for angiogenesis, we used a tube formation assay with HUVECs, as published previously.[16] Passages 8-15 were used for experiments. HUVECs were maintained in EGM™-2 media (Lonza #CC-4176) and passaged as mentioned above.[14] We used Angiogenesis μ-slides (Ibidi #81501), filled

with Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher #A1413202). 10 µL matrigel was inserted into the lower well of the µ-slide. 45-60 minutes later, 3000-5000 HUVECS were seeded per well, with the addition of the drugs or vehicle (DMSO) in a total volume of 50 µL. After 2-4 hours, pictures were taken with a 2x enlargement. Images were analyzed using Image J® and the Angiogenesis Analyzer of the Carpentier Lab.[17] Values for length, number of junctions and number of master junctions were recorded. We used a live-dead assay (ThermoFisher, #L3224), according to manufacturer's protocol.

(Myo)fibroblast differentiation assay

Human fetal cardiac fibroblasts (hCFs) were used in these experiments, coming from fetal donors, isolated using an in-house protocol. In short, heart tissue was dissolved and plated for 2h on regular plastic culture dishes after subtraction of Sca-1$^+$ cells. All adhered cells were considered hCFs and kept for further passaging. hCFs were maintained in DMEM (Gibco #41965-039), supplemented with 10% Fetal Bovine Serum (Gibco #10270-160) and 1% Penicillin/Streptomycin (P/S) (Gibco #15140-122) and passaged as mentioned above.[14]

hCFs were plated in 12-well plates (Corning, #3512) coated with 0.1% gelatin and starved for at least 3 hours in DMEM, supplemented with 2% FBS, before being stimulated with TGF-b (end concentration 5ng/ml, Peprotech 100-21c) and comedication or DMSO(vehicle) for 24 hours. Afterwards, the cells were either lysed or stimulated with TGF-b and the drugs for a subsequent 24 hours. Lysing and RNA extraction was done using the Nucleospin RNA Isolation Kit (Macherey-Nagel, #740955). cDNA was made, using the iScript Advanced cDNA synthase kit (Bio-Rad #1725038). qPCR was performed on C-1000 Touch Thermo Cyclers (Bio-Rad), using SYBR Green Iq Supermix (Bio-rad #1708880). Primer pairs are listed in Supplementary Table 1.

Antigen-response assay

Human Peripheral Blood Mononuclear Cells (hPBMCs) were isolated using Ficoll gradients from 7 different donors. PBMCs were cultured in RPMI media (Gibco, #61870) (supplemented with 1% P/S + 10% FBS). For stimulation, a combination of IL-2 (120U/ml, BD Pharmingen, #554603) and PMA (0.123ng/ml, Sigma, P8139) was used. Cells were co-cultured for 10 days in 48-wells plates (Corning, #3548) ($2.5*10^5$ cells/well) together with hMSCs ($5.0*10^4$ cells/well). After 10 days, supernatant was collected. Comedication was added the first day in fresh medium (in a 10x solution), without replacing the original media. A human isotyping 6-plex (Biorad # 171A3103M) was used to measure the levels of total IgA, IgM and 4 subclasses of IgG in the supernatant of the co-cultures using a luminex-200 instrument (Bioplex-200). The luminex assay was performed according to manufacturer's protocol.

Statistical analysis

For the meta-analysis dataset, we used univariable meta-regression. For the *in vitro* data, we used a two-way ANOVA, with the addition of the factor 'experiment', which reduced the *between experiment* variance, while maintaining *within experiment* variance for our primary analysis. Data for meta-regression is depicted as a mean with 95% confidence intervals. Data for *in vitro* assays is depicted as mean ±SD. For all data analyses, R was used with the addition of the meta and xlsx packages.[18-20]

Results

Meta-regression suggests effect of comedication on cell therapy efficacy in large animal MI studies

Meta-regression for the absence or presence of clinically prescribed drugs (used throughout the preclinical study), reveals a significant decrease for cell therapy efficacy when any of the drugs are present, going from an ejection fraction gain of 8.8% (95%CI 7.5 - 10.0) to 2.4% (95%CI -2.0 – 6.8) over placebo therapy (Figure 1A, p=0.007). The standardized mean difference for end diastolic volume is significantly reduced from -10.7 (95%CI -14.3 - -7.0) to 1.6 (95%CI -8.8 – 11.9) in the presence of comedication (Figure 1B, p=0.03), while the standardized mean difference for end systolic volume showed a trend for reduction from -8.9 (95%CI -12.2 - -5.6) to -0.8 (95%CI -9.2 – 7.6) (Figure 1C, p=0.08).

Drugs used throughout the studies from this dataset were aspirin (n= 6 studies), clopidogrel (n= 3), β-blockers (n=3) and ACE/angiotensin inhibitors (n=1). Statins were not used in any of the studies. No studies used the combination of all drugs classes. No study mentioned the use of these to control for the clinical presence in the disease under study.

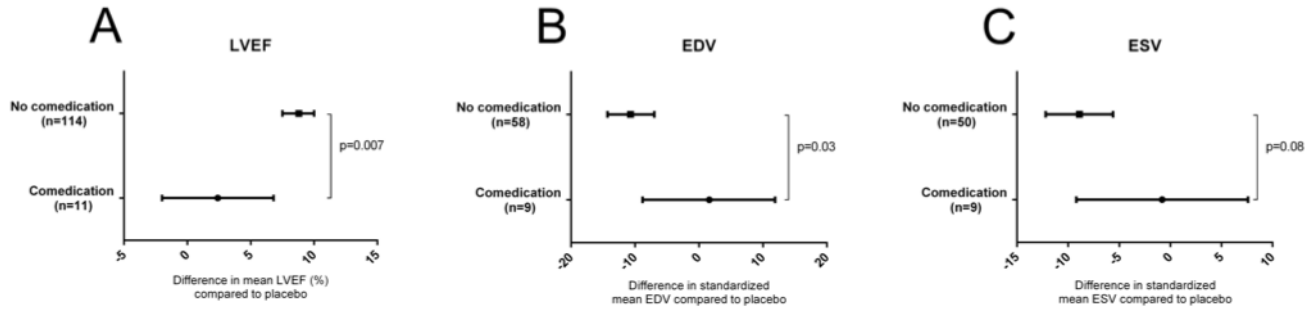

Figure 1. Meta-regression comparing studies either using one or more of clinically prescribed MI drugs compared to studies using none, for (A) left ventricular ejection fraction, (B) end diastolic volume and (C) end systolic volume. n= number of comparisons.

HUVECs are affected by comedication and hMSCs

Comedication shows a concentration-dependent positive effect on tube formation in our tubular assay (Figure 2A-H). With the addition of conditioned media from hMSCs, the tubular formation is increased even more, while the addition of the drugs still seemed able to influence the assay positively for total junctions (Figure 2I, p= 0.005), master junctions (Figure 2J, p=0.02), and the common standardization measure length/junction (Figure 2K, p<0.001). For added therapeutic value of hMSC-conditioned media, only the DMSO comparison was statistically significant (post-hoc p-value=0.001). We transformed the length/junction outcome to a ratio for conditioned vs non-conditioned samples, visualizing a decreased additional benefit of hMSCs in a concentration-dependent matter (Figure 2L, p=0.07). To confirm a direct effect on tubular formation and exclude changing in cellular viability, we also performed a live-dead staining on the multiple conditions, showing no difference between our experimental groups (Figure 2M-P).

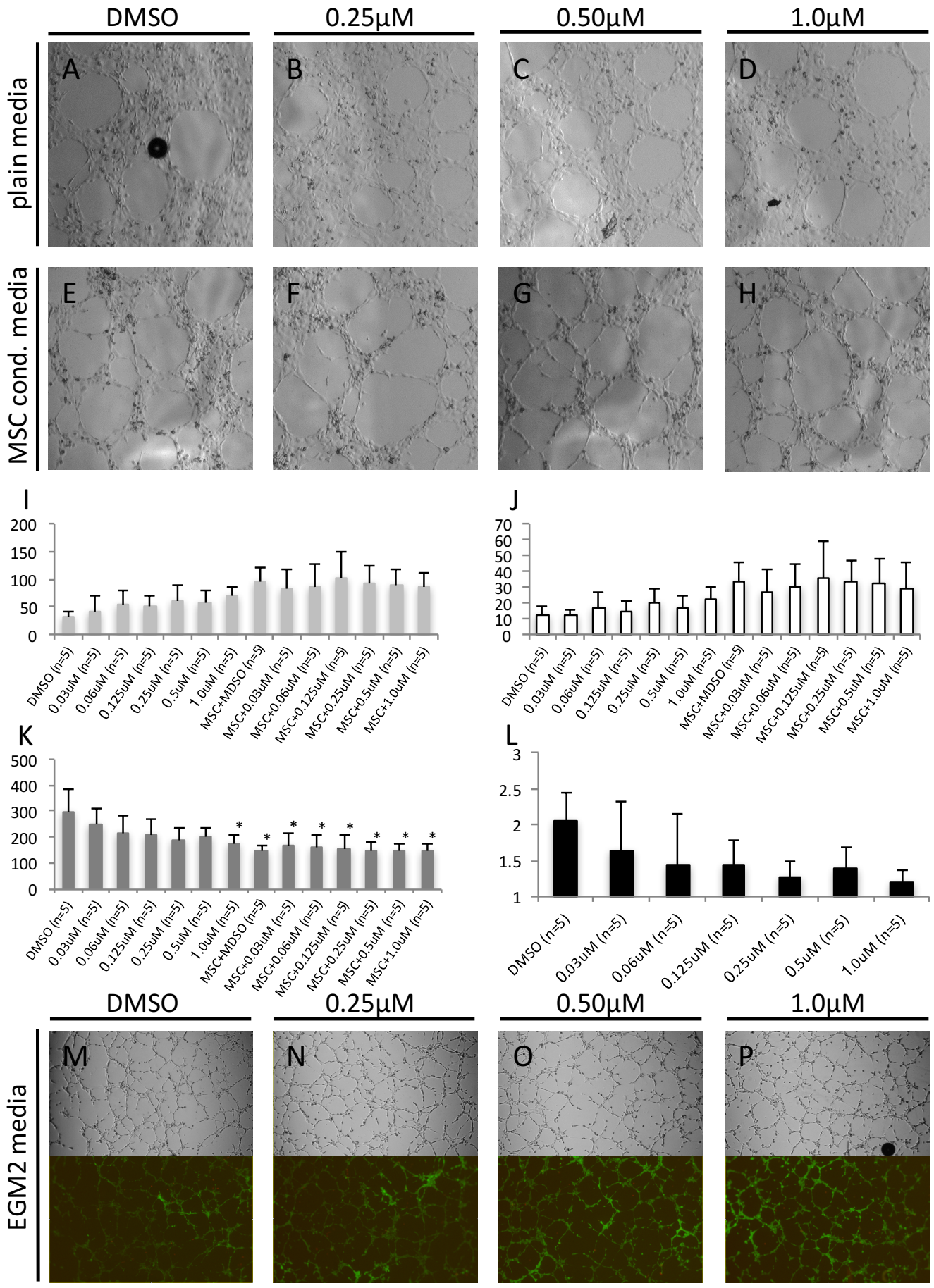

Figure 2. Comedication and hMSCs influence tube formation in HUVECs and affect the therapeutic window. (A-H) representative pictures of tube formation assay. (I) number of junctions (p=0.005). (J) Number of master junctions (p=0.02). (K) Total length/no. of junctions (p<0.001). (L) ratio of MSC-cond vs non-cond samples for the length/no. of junctions measurement. * significant compared to DMSO in post-hoc testing.

Immune response is reduced by hMSCs and unaffected by comedication

By isolating PBMCs and stimulate them with IL-2 and PMA *in vitro*, we are able to measure their response through immunoglobulin excretion. The addition of comedication in different concentrations did not change their excretion patterns (Supplementary Figure 1). hMSCs suppressed the formation of different immunoglobulins after IL-2 and PMA stimulation (Figure 3A-F). The addition of comedication did not change this immunosuppressive effect of hMSCs.

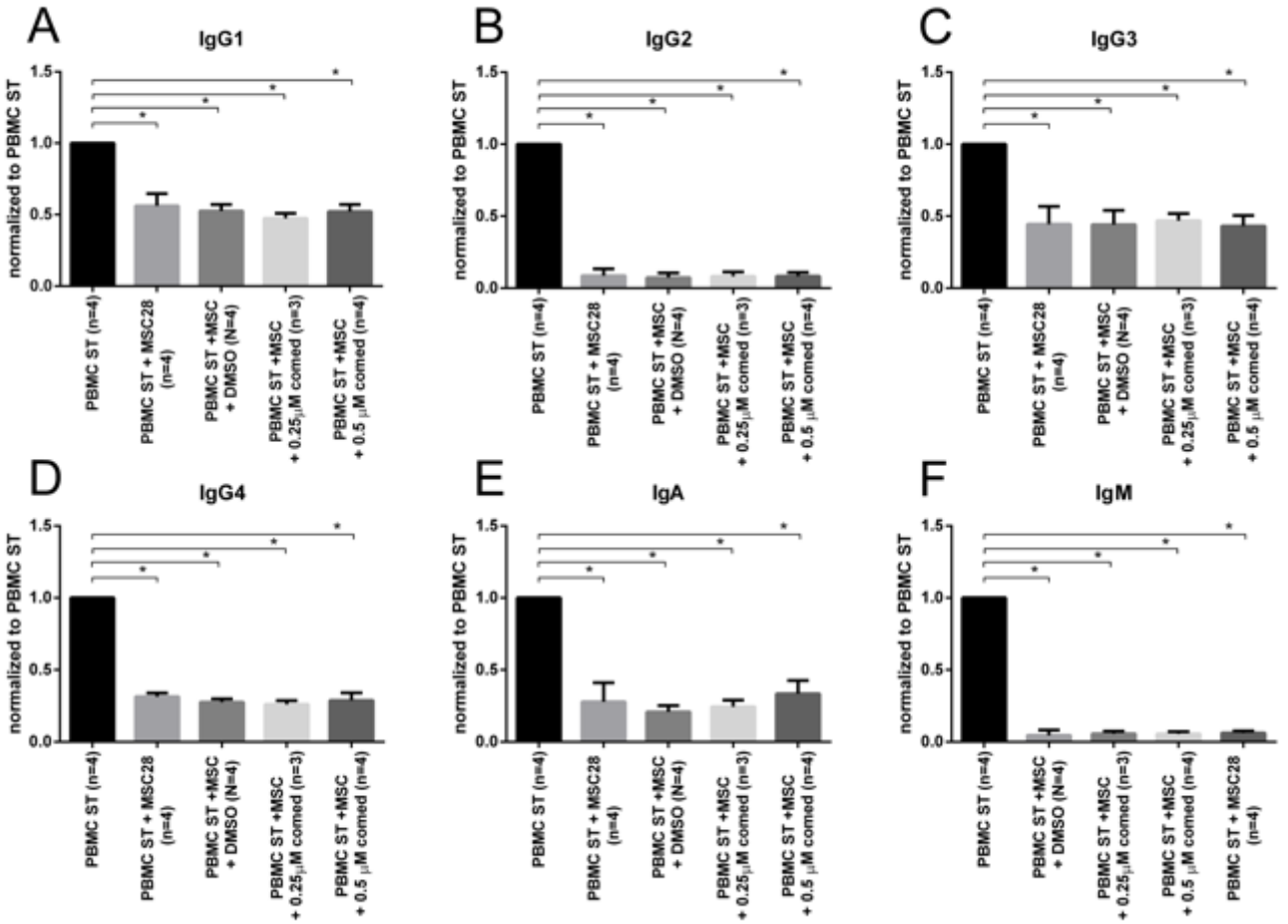

Figure 3. Antibody production is suppressed through addition of hMSCs, but not by comedication. (A) IgG1 ($p<0.001$), (B) IgG2 ($p<0.001$), (C) IgG3 ($p<0.001$), (D) IgG4 ($p<0.001$), (E) IgA ($p<0.001$) and (F) IgM $p<0.001$). * Post-hoc Tukey test <0.001

Myofibroblast differentiation seems modified by comedication

Our myofibroblast differentiation assay showed a trend towards reduction in myofibroblast gene expression, when incubated with comedication, compared to the DMSO control (Figure 4A-B). αSMA showed non-significant reductions after 24 ($p=0.14$) and 48 hours ($p=0.29$) of incubation with comedication and TGF-β, while only the 48h samples of Col1a2 showed the same non-significant trend ($p=0.18$).

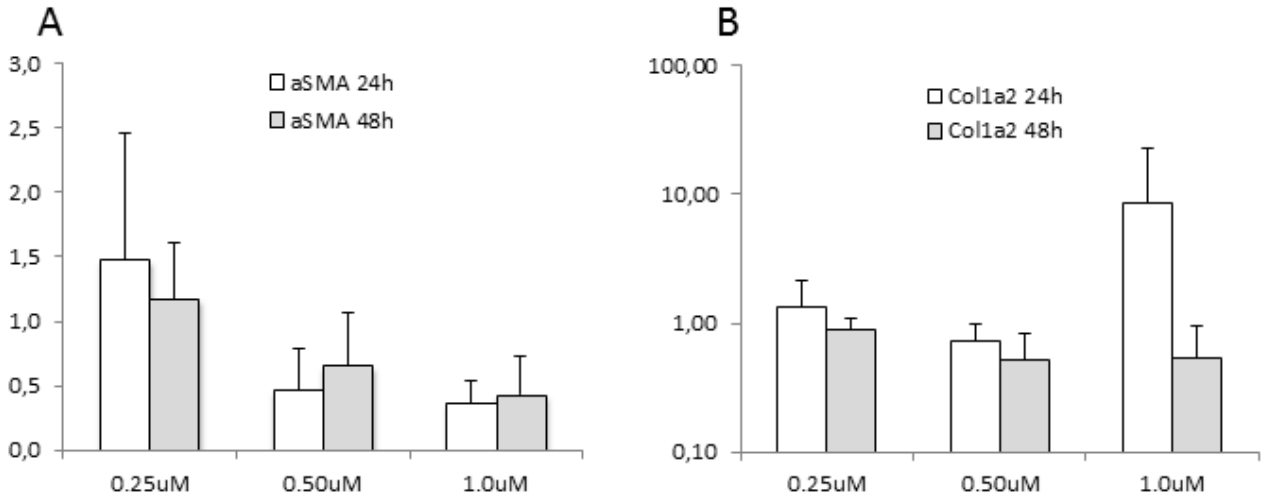

Figure 4. Myofibroblast differentiation experiments. Graphs show the fold change compared to the DMSO control for the genes αSMA (A) and Col1a2 (B). n=3 experiments for both time points for all samples.

hMSC-function seems unaffected by comedication

As cell therapy products are biological interventions, the therapeutic itself can be influenced by its environment too. Therefore, we also tested hMSC function in the presence of our clinically prescribed drugs. hMSC proliferation does not seem affected by the addition of comedication (Figure 5A-B), while also viability is not an issue in the presence of these drugs (>95%, no differences between groups, data not shown). As hMSCs are thought to have a paracrine mode of action, we also investigated the excretion of growth factors, when incubated with comedication. Using a growth factor and cytokine array, we also show that the composition of excreted growth factors mostly remains unchanged when hMSCs are incubated with varying concentrations of the drugs (Figure 5C). Excretion of VEGF, HGF, and TGF-β stayed stable across samples (Figure 5D-E,H). The cytokines IL-6 and IL-8 seemed increased upon incubation with comedication, compared to the DMSO control (Figure 5F-G).

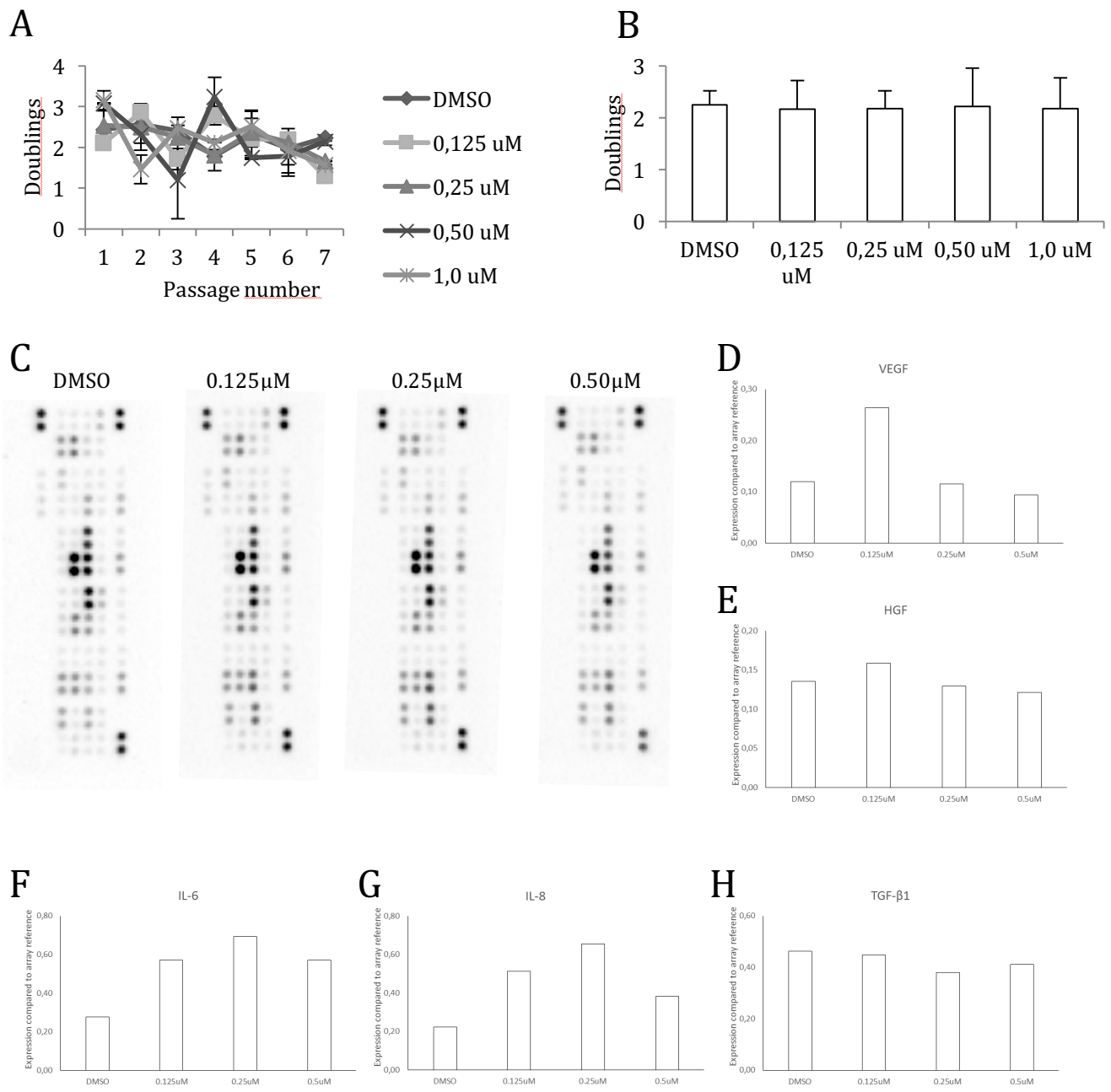

Figure 5. hMSC function is unaffected by comedication. (A) Doublings per passage. (B) average no. of doublings over all passages. (C) growth factor array. (D) VEGF release, as analyzed on array. (E) HGF release, as analyzed on array. (F) IL-6 release, as analyzed on array. (G) IL-8 release, as analyzed on array. (H) TGF-β release, as analyzed on array.

Discussion

In the current work, we have tried to explore the effect of clinically prescribed MI medication on cardiovascular disease assays and cell therapy efficacy in MI. Here, we show that large animal MI studies, using any of the clinically prescribed MI drugs, show a marked reduction of cell therapy efficacy. As subsequently shown, a number of commonly used assays seem influenced by the combination of these drugs in a concentration-dependent matter. These results support our hypothesis that these drugs can affect efficacy readouts and thereby might explain current difficulties to translate cell therapy to the clinic. Only testing new therapies on the background of

clinically prescribed drugs in clinical trials might prove to be late, counterintuitive, inefficient and an uneconomical use of research money.

Regular comedication affecting important processes

There is ample research on the effects of MI comedication on certain beneficial and detrimental cardiovascular processes. Many of these drugs have been shown to affect angiogenesis[21], cell-death and apoptosis[8,22,23], fibrosis[24,25], immune responses[6,26,27], and other processes of cardioprotection.[9,28-31] Interestingly, combinations of aspirin and statins have also shown dismantling instead of synergistic patterns on cardioprotection.[10,32] Direct effects on the bone marrow and its circulatory offspring have also been reported, showing increase of reparative functions.[33-35] Again, this could be one of the mechanisms causing increased cardiac repair *in vivo* and less 'need' for new therapies. Importantly, if these therapies affect other processes than the ones they are officially designed for (e.g. angiogenesis) this might also seriously affect considerations for therapy in other diseases like cancer. For statins for example, researchers are still not agreeing on a possible protective or negative effect on cancer mortality.[36]

Comedication affecting cell therapy efficacy

Minimal effort has been put in studying the specific effects of comedication in combination with cell therapy. A recent study reported enhanced isolation and improved phenotypes of cardiosphere-derived cells if patients were treated with β-blockade.[37] Studies using myoblasts and endothelial progenitor cells both showed increased effects of their therapy when incorporated with either ACE-inhibition alone or combined with β-blockade.[38,39] Both small and large animal studies also showed the increased efficacy of the combination of statins and cell therapy compared to the groups using either one.[40,41] In all papers, the beneficial 'added effect' of cell therapy compared to a control seemed to decrease when comedication was incorporated, which is in line with our current data.

The added value of cell therapy

Interestingly, where some processes seemed influenced by comedication in our *in vitro* assays, the antigen production of immune cells was not affected by this, where hMSCs were able to significantly reduce antigen production in both presence and absence of comedication. hMSCs are known to modulate the immune response after myocardial damage, which has been proposed as one of the cell's mode of action.[42,43] It might be this mechanism that can still have a beneficial effect in cell therapy trials, as it might not be hampered by the presence of comedication. Furthermore, we don't know if cell therapy affects other physiologic processes. Future *in vivo* animal studies will tell us if these effects are still present and if external validity of cell therapy studies can be increased through addition of these drugs.[44]

Future research and recommendations

In light of the demonstrated potential effects on processes like angiogenesis and fibrosis it is likely that comedication also influences the disease and efficacy of new therapeutics in *in vivo* preclinical studies and regular clinical care. Additional experiments are currently ongoing, including *in vivo* studies an in-depth investigation of the individual drugs responsible for the effects we see and their specific mechanisms. As these drugs are known to affect physiology, next to the described isolated

mechanisms *in vitro*, we might expect more and other effects from these in the *in vivo* situation. Currently we are setting up these models, with specific interest for long-term feasibility of their usage (data not shown). To accurately mimic the clinical situation in a standardized way might be an important step for our *in vivo* confirmatory studies. As these drugs seem to have such a striking effect on core processes affecting cardiac repair and are relatively easily incorporated in assays, we will not be able to disregard them in confirmatory stages of new therapeutics if we want to study realistic therapeutic windows. Of course, translatability is not only confined to drugs, but also involves risk factors and other disease specifics.[45]

The used *in vitro* assays are best performed in human cells as any effect can be most likely translated to the clinical situation. Our *in vivo* murine studies might have different mechanisms of these drugs through non-conserved differences between mice and man, in for example the immune system, yet might still be relevant for the effects that are evolutionary conserved.[46] If the shown effects are applicable in a murine *in vivo* setting, this might be the most cost-effective stage to regularly incorporate these drugs for confirmatory purposes, maybe in parallel with first large animal studies.

In an era of evolving therapeutics, we are also dealing with evolving ischemic disease, of which part can be attributed to better and other treatment strategies. Our research models need to evolve alongside of that. Not only in the cell therapy field, but also in guideline-implemented therapies like ICD therapy, trials have not reached primary endpoints, possibly because of low event rates and changing disease spectra.[47] The same phenomenon might be happening in our clinical cell therapy trials too, which were initially powered on highly promising large animal studies.[13] It is therefore crucial to most accurately mimic those specific circumstances in preclinical situations, for most accurate testing and translation of new therapeutics in earlier stages of research. We might be able to pull the plug on new compounds earlier, saving resources for more promising research. Ideally, this will lead to more accurate expectations, more realistic power calculations for clinical trials and ultimately most efficient translation of the right therapeutics that stand a chance against the care that already has been improved so much over the past decades.

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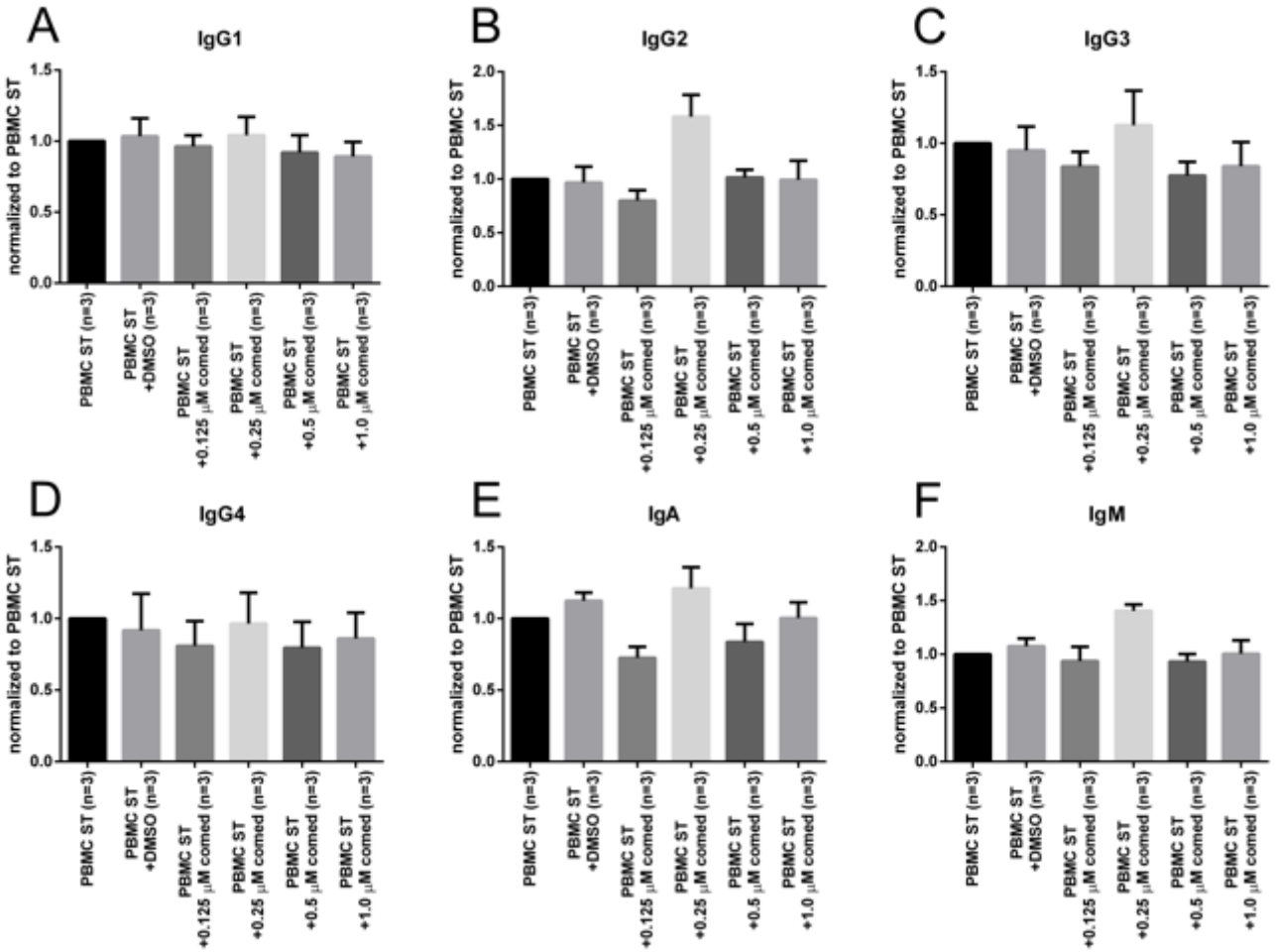
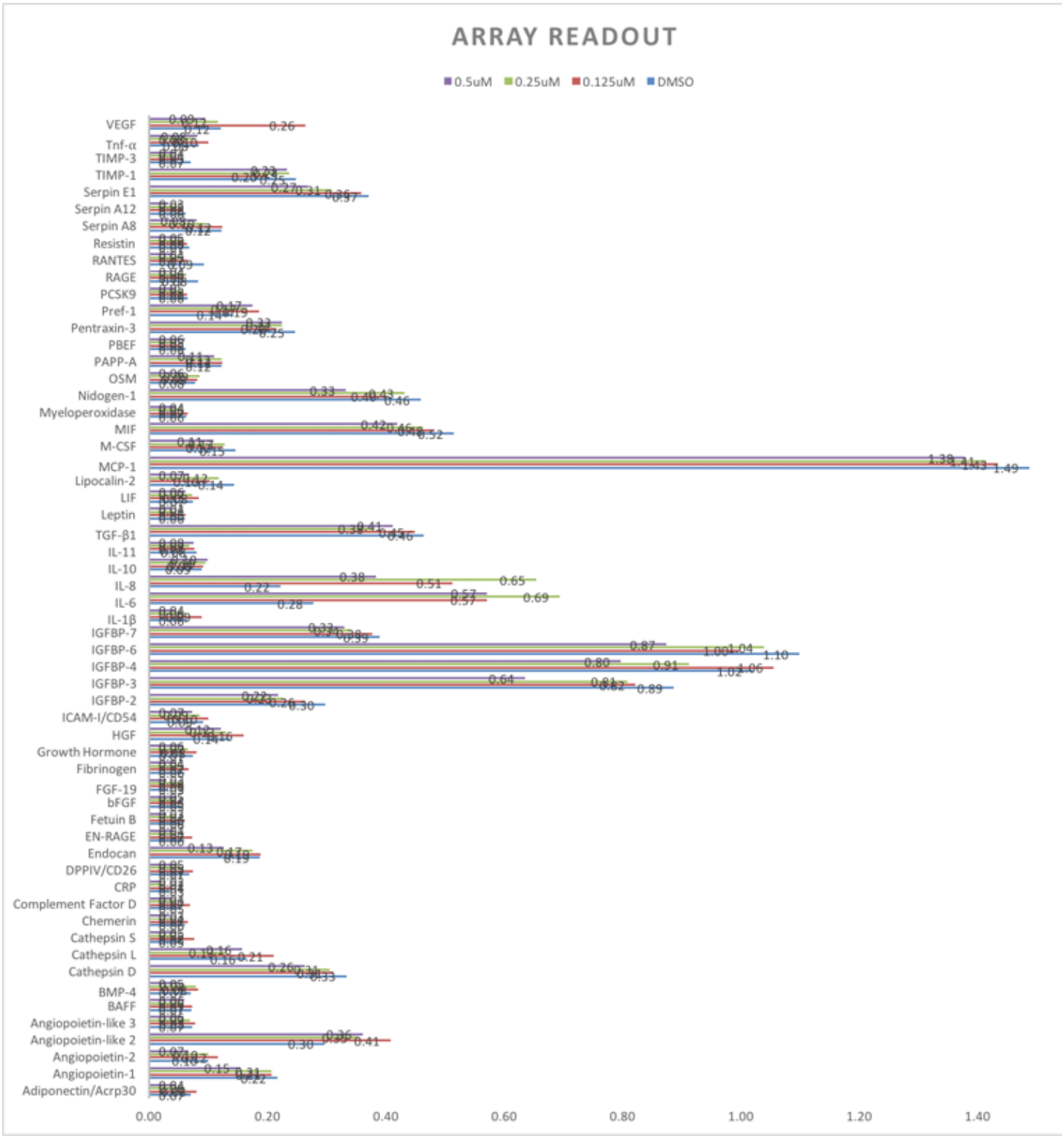
Oligo name	Oligo sequence (5' to 3')
hGAPDH F	ACAGTCAGCCGCATCTTC
hGAPDH R	GCCCAATACGACCAAATC
hACTA2 F	AGCCCAGCCAAGCACTG
hACTA2 R	CAAAGCCGGCCTTACAGA
hCol1a2 F	GGCCCTCAAGGTTTCCAAGG
hCol1a2 R	CACCCTGTGGTCCAACAACTC

Supplementary Table 1. Primer pairs for qPCR



Supplementary Figure 1. The addition of comedication does not affect the excretion of immunoglobulins of PBMCs. (A) IgG1 (p=0.55), (B) IgG2 (p=0.11), (C) IgG3 (p=0.27), (D) IgG4 (p=0.41), (E) IgA (p=0.15) and (F) IgM (p=0.16).



Supplementary Figure 2. All expression patters from cytokine array analysis for hMSCs treated with DMSO (vehicle), 0.125 µM, 0.25µM or 0.50 µM. Most excreted cytokines are excreted similarly across samples, except for Il-6 and Il-8.

6

Cardiac stem cell treatment in myocardial infarction; protocol for a systematic review and meta-analysis of preclinical studies

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ABSTRACT

Cardiac-derived stem or progenitor cells (CSCs) have emerged as a possible therapeutic intervention for myocardial infarction, potentially ameliorating the devastating effects caused by inadequate blood flow to the heart. The first human clinical trials using these myocardial-derived cells have recently started, but scientific controversy exists regarding the efficacy and origin of some of these stem cells in the pre-clinical animal models. Systematic review of the current literature on CSCs in ischemic cardiomyopathy can provide useful additional information on the use of CSCs in pre-clinical trials. By combining all available data, we can adequately compare the different types of cells being used and possibly identify factors that influence cardiac stem cell therapy in general. This protocol provides a thorough description of the methodology that will be used in our systematic review and meta-analysis of all pre-clinical animal studies involving cardiac stem cell treatment for ischemic cardiomyopathy.

General

The structure of this protocol is adapted from and based on the Systematic Review Protocol for Animal Intervention Studies.[1]

Title of the systematic review

Cardiac stem cell treatment in ischemic cardiomyopathy; a systematic review and meta-analysis of pre-clinical studies

Stage of the project at time of protocol submission

Stage of process	Started	Completed
Preliminary searches	Yes	Yes
Piloting study selection	Yes	Yes
Formal screening with final search criteria	Yes	No
Data extraction from included papers	Yes	No
Quality Assessment	No	No
Data analysis	No	No
Manuscript writing	No	No

Background

Cardiovascular diseases are the leading causes of death in current medical practice, with more than 7 million people dying from ischemic heart disease in 2012.[2] Because therapy for coronary heart disease is ever-evolving and improving, chronic disease burden is increasing due to the aging population and longer survival after an initial ischemic event.[3] Stem cell therapy has been proposed as an additive therapeutic after myocardial infarction (MI), aiming at stimulating or contributing to regenerative effects.[4] In particular, cardiac-derived stem or progenitor cells (CSCs) hold great potential as they already originate from the heart, can differentiate into all cardiovascular lineages and have the potential to stimulate regeneration of the heart through several mechanisms.[4] Different CSC types have been discovered over the past decade; the c-kit+ CSC, the cardiosphere and cardiosphere-derived cell (CDC), the sca-1+ CSC, the islet1+ CSC and the side population (SP) cells have all been isolated from adult myocardial tissue.[5-9]

Human trials (SCIPIO for c-kit+ CSCs and CADUCEUS for CDCs) have been started recently and the initial results look promising.[10,11] Nonetheless, researchers have recently questioned the origin of the cardiac stem cell and its potential.[12] With not all pre-clinical studies reaching positive outcomes for the CSCs, a solid and complete overview of all pre-clinical studies conducted with CSCs is still lacking. By combining all available data, we can make accurate comparisons between the different CSC types, which are currently being used. Furthermore, all pre-clinical studies combined will provide us with additional information on study design and effectiveness of CSCs in MI models and might help us in optimizing our treatment strategies and translation towards clinical studies. Additionally, performing a meta-analysis of these studies will provide information on possible factors that influence efficacy of CSCs in animal models of MI.

Objectives of the systematic review and meta-analysis

Specify the disease / health problem of interest

In the current study, myocardial infarction is defined as ischemia resulting in permanent damage to the myocardium, caused by the disruption of adequate blood flow. In real life this is usually caused by obstruction of one or more coronary arteries due to rupture of an instable atherosclerotic lesion.[13] In pre-clinical models mechanical obstruction of one of the coronary arteries is most commonly used.

Specify the population /species studied

Research regarding CSCs as a therapeutic agent to stimulate cardiac performance started in 2003.[5] Since then, several studies have been published in many different animal models. We will include all placebo-controlled pre-clinical studies using the following animals; mice, rats, guinea pigs, rabbits, goats, sheep, dogs, pigs.

Specify the intervention/exposure

The intervention of interest is the administration of CSCs. A cardiac stem or progenitor cell is defined as a stem cell, showing (some degree of) clonogenicity, residing in the adult heart with the ability to commit to cell types of the cardiovascular lineage (cardiomyocytes, smooth muscle cells and endothelial cells).[5-9] 5 different CSCs harvested from the adult heart have been repetitively identified and will therefore be included in our systematic review:

- C-kit+ CSC[5]
- cardiosphere / CDC[6]
- Sca-1+ CSC[7]
- Islet-1+ CSC[8]
- SP cell[9]

Specify the control population

Studies will be included when using placebo treatment; phosphate buffered saline (PBS), vehicle solution (e.g. culture medium) or cells of another origin as a control. Sham animals or affected animals without administration of a placebo will be excluded.

Specify the outcome measures

Primary outcome: Ejection fraction (EF)

Secondary outcome: end systolic volume (ESV), end diastolic volume (EDV), wall thickness (WT), fractional shortening (FS), infarct size (IS) (per area at risk (IS/AAR) and per left ventricle (IS/LV))

State your research question

What is the effect of CSC therapy in myocardial infarction animal models when compared to placebo-treated controls?

METHODS

Search and study identification

Identify literature databases to search
Based on previous experience we chose to search the Pubmed and Embase databases.

Define electronic search strategy (final searches conducted on 5-11-2014)
Pubmed:
("cardiac stem cell" OR "cardiac stem cells" OR "cardiac progenitor" OR "cardiac progenitors" OR "cardiomyocyte progenitor" OR "cardiomyocyte progenitors" OR cardiosphere OR cardiospheres OR CMPC OR CSC OR CPC OR CDC)

AND (cardiac OR heart OR myocardial OR infarction OR ischemic)

AND (pig OR dog OR canine OR sheep OR goat OR porcine OR swine OR ovine OR mice OR mouse OR rat OR rats OR murine OR rabbit* OR "guinea pig")

Embase:
('cardiac stem cell' OR 'cardiac stem cells' OR 'cardiac progenitor' OR 'cardiac progenitors' OR 'cardiomyocyte progenitor' OR 'cardiomyocyte progenitors' OR cardiosphere OR cardiospheres OR CMPC OR CSC OR CPC OR CDC)

AND (Cardiac OR Heart OR myocardial OR infarction OR ischemic)

AND (pig OR dog OR canine OR sheep OR goat OR porcine OR swine OR ovine OR mice OR mouse OR rat OR rats OR murine OR rabbit* OR 'guinea pig')

Other sources for study identifications
Reference lists of included studies and relevant reviews.

Study selection procedure

Define screening phases and number of observers (2 observers per phase)
1. Title/abstract screening (PPZ / AV)
2. Full-text screening (PPZ / AV)

In both phases the two observers try to reach consensus on inclusion by discussion. In case of no consensus by the two primary observers a third reviewer (JS) is consulted.

Study selection criteria

Type of study design
Inclusion: placebo-controlled randomized trial, placebo-controlled (cohort) study

Exclusion: review, editorial, case report, case series, protocol paper, study without placebo-control group

Type of animals / population (e.g. age, sex, disease model)

Inclusion: Any animal MI model by coronary occlusion >10 minutes (rodents) or >30 minutes (non-rodents) by ligation, balloon occlusion, micro-embolization, coil embolization, sponge embolization or any other temporary or permanent occlusion method of an animal's coronary artery. Cut-off points for rodents and non-rodents, respectively, are 10 and 30 minutes based on literature on myocardial stunning and pre-conditioning, which we want to exclude.[14,15]

Exclusion: fetal ischemia models (in utero), ischemia less than 10 minutes for rodents or less than 30 minutes for non-rodents. Studies that perform co-interventions will be excluded.

Type of intervention

Inclusion treatment: CSCs as previously defined.

Exclusion criteria: Since we solely want to determine the effect of CSCs that naturally reside in the myocardium, we choose to exclude genetically modified cells, pre-treated cells, cells in/on scaffolds/patches/beads, fully differentiated cardiomyocytes, embryonic-derived cardiac progenitors and treatment with cell-derived material like conditioned medium or extracellular vesicles.

Outcome measures

Studies will be included in the analysis if they reported the primary outcome measure EF or a combination of both individual ESV and EDV (from which the individual and mean EF can be calculated). If a study uses an imaging modality generally used for EF measurements, but did not report these, authors will be emailed to ask to provide possible data. If a study mentions quartiles instead of means in combination with a standard deviation (SD) or standard error of the mean (SEM), authors will be emailed to ask to provide the raw data or means and SDs or SEMs. Authors will also be emailed if the number of animals per group is not stated and asked to provide the information.

Exclusion criteria: Studies will be excluded if data on EF or the number of animals per group could not be obtained (either through extraction from the paper or after repetitive email contact).

Language restrictions

Inclusion: English

Exclusion: Any language other than English

Publication date restrictions

We did not include any date restrictions in our search. We will discard papers published before 2002 since discovery of the first CSC being used for therapy was published in 2003.[5]

Other

Inclusion criteria: full text original papers

Exclusion criteria: congress abstracts

In case experimental groups and data are used repeatedly in different studies (e.g. to answer different hypothesis), we will include these data only once.

Order of priority exclusion criteria per screening phase

Order for title/abstract screening

1. No CSC treatment
2. No MI
3. No original data (e.g. review, editorial, etc.)
4. No animal study
5. In utero ischemia model

Order for full-text screening

1. No full-text paper
2. No CSC treatment
3. No MI
4. No original data (e.g. review, editorial, etc.)
5. No animal study
6. In utero ischemia model
7. No imaging modality suitable for EF measurement
8. No placebo-control
9. Number of animals per group not stated

Study characteristics to be extracted

One reviewer will extract study characteristics and all data input will be checked by another reviewer in the database.

Study ID

DOI; first author; corresponding author; journal; publication year; source of funding.

Study design

The number of animals per group will be extracted. If the exact number per group is not mentioned (but for example only a range) the lowest number of animals will be used for data analysis.
CSC treatment, when reported as either the primary treatment or as a control treatment when testing for improved therapy, will be extracted. Information on the use of immunosuppression or immune-compromisation will be extracted.

Animal model

Animal type (rodent or non-rodent); species; breed / strain; sex; age; weight; method of induction of injury (ligation, balloon occlusion, embolization); ischemia model (permanent or ischemia-

reperfusion), duration of occlusion, comorbidity.

Intervention characteristics

Type of CSC; cell dose; time of delivery relative to time of induction of ischemia and reperfusion; route of delivery; duration of follow-up and time of functional cardiac assessment after cell delivery; cell characteristics (2D/3D culture, autologous/syngeneic/allogeneic/xenogeneic, comorbidity); CSC-group used as primary intervention of the study or as a control for another treatment. In case of multiple timepoints the latest time point will be included for uniformity and since this has the most clinically relevant implication.

Outcome measurements and data collection

1. Method of functional outcome assessment
2. Left ventricular EF as percentage
3. ESV and EDV in mL
4. IS/AAR and IS/LV as percentage
5. WT in mm
6. FS as percentage

All data will be extracted as a mean with SD or SEM for database input.

Risk of bias assessment

Risk of bias is assessed based on the CAMARADES checklist[16] which includes the following criteria:

1. Publication in a peer reviewed journal
2. Reporting of random allocation
3. Reporting of blinding of the operator
4. Reporting of blinded assessment of outcome
5. Use of comorbid animals
6. Reporting of a sample size calculation
7. Reporting of compliance with animal welfare regulations
8. Reporting of a potential conflict of interest

Moreover, attrition bias will be measured using a specific part of the SYRCLE's risk of bias tool.[17]

Methods of data extraction and retrieval

Data is preferably extracted from either text or tables in the results section of the manuscript of interest. When the data is not available in text or tables, data will be extracted electronically from available graphs using the Image J® software, version 1.48 (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015). If an imaging modality capable of measuring EF is being used, with no mentioning in the manuscript of an EF, authors will be contacted for the data by email. In case of no response after four weeks, including a reminder, manuscripts will be excluded from the analysis. If only individual data is present, mean and SD will be calculated from these values.

Data-analysis and –synthesis

Data gathering
All data will be inserted in the CAMARADES database (data available upon request).[18]

Data combination
Data will be combined in a systematic review, forest plot and subsequent meta-analysis.

Specify if and when data combination is appropriate
We expect to include over 40 studies. We choose a minimum number of 25 studies to be included; we need at least 25 studies to make sure we can adequately determine publication bias and conduct our meta-analysis.[19]

First we will pool all data for our general outcomes. We expect to encounter differences in effect between rodents and non-rodents (e.g. large animals) for our analyses, since this has been reported previously in research involving therapeutic modalities in pre-clinical cardiac disease models.[20] Therefore, we will stratify these groups upfront and pool these data separately for additional meta-regression analyses.
We expect the different ischemia models and outcome measures to be uniform and widely used in the same way. Furthermore, all three major CSC (c-kit+, CDC and Sca-1+) types have shown comparability in cell characteristics when cultured under the same circumstances.[21] Therefore, we think it is feasible to pool our data for a combined analysis for each separate outcome measurement.

Our interest is in parameters that influence our primary outcome in study, animal and/or cell characteristics. Direct comparison in our eyes is feasible when groups contain 5 or more studies. To explore sources of heterogeneity in our included studies we will conduct a meta-regression; significant predictors will be further investigated based on the outcome of the meta-regression. The number of parameters, tested by meta-regression, is 1 parameter for every 10 included studies. For the primary outcome (EF) no correction will be applied, with a p-value <0.05 regarded as a significant difference. For all secondary outcome measures we will correct for the number of parameters tested with a Bonferroni-Holm correction.

If meta-analysis is feasible

Specify effect measures to be used
We expect the values to be heterogeneous with regard to animal sizes and imaging modalities for our study. Most of our outcomes are measured in percentages/ratios, so for EF, IS and FS we will use raw mean difference as our effect measures since these modalities are already corrected for size of the animal. Since animal size will vary between studies, absolute measures (ml, mm) will vary as well. In order to combine these data, a standardized mean difference analysis will be performed for ESV, EDV and WT. Depending on the reported values, we will extract the reported value with the additional standard error of the mean (SEM) or standard deviation (SD). Studies

reporting median will be excluded.

Outcome measurements:

1. EF: raw mean difference
2. EDV / ESV: standardized mean difference
3. IS/AAR / IS/LV: raw mean difference
4. WT: standardized mean difference
5. FS: raw mean difference

Specify which study characteristics will be analyzed as possible sources for heterogeneity

1. Cell type (c-kit+ CSC, cardiosphere/CDC, Sca-1+ CSC, Islet1+ CSC, SP cell)
2. Immunosuppression (yes/no)
3. Cells being used as control or ultimate treatment
4. Cell characteristics upon administration
    a. 2D or 3D cultured
    b. Comorbidity (diseased vs. healthy)
    c. Autologous vs. syngeneic vs. allogeneic vs. xenogeneic
5. Animal characteristics
    a. Age of recipient animal
    b. Sex of recipient animal (male/female/mixed/unknown)
    c. Animal species
    d. Strain or breed within species
6. Timing of therapy
7. Timing of assessment
8. Randomization (yes/no)
9. Blinding
    a. Allocation concealment (yes/no)
    b. Assessment of outcome (yes/no)

Specify statistical model of analysis

Our data will be heterogeneous since we include studies using different study designs (i.e. animal species, cell type) and therefore we will use a random effects model for analysis. We will quantify the extent of heterogeneity present in our dataset by determining the Tau^2 and I^2 statistics.
Statistical analysis will be performed using Stata Statistical Software: Release 13 (College Station, TX: StataCorp LP).

Methods for assessing risk of publication bias

Risk of publication bias will be assessed using funnel plotting and Egger's regression analysis.[22] Missing studies will be identified using Tweedie and Duval trim and fill analysis.[23]

Sensitivity analysis

A sensitivity analysis will be performed for time of outcome measurement. Clinically the latest timepoint seems most relevant to us. However, there might be considerable variation in the

timepoints of outcome assessment. Therefore, we will compare these outcomes to outcomes closest to the commonly used timepoint (in case of multiple measurements) in our studies (most likely around 3-4 weeks).

Expected possible limitations of this systematic review

The data might be too heterogeneous to make adequate subgroups (>5 studies) and to do an adequate meta-regression. It could also be that the number of articles will be less than expected; again, a meta-analysis might not be feasible in that case.

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The authors declare that there are no conflicts of interest.

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7

Cardiac stem cell treatment in myocardial infarction; a systematic review and meta-analysis of preclinical studies

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ABSTRACT

Rationale

Cardiac stem cells (CSC) therapy has been clinically introduced for cardiac repair after myocardial infarction (MI). To date there has been no systematic overview and meta-analysis of studies using CSC therapy for MI.

Objective

Here, we used meta-analysis to establish the overall effect of CSCs in preclinical studies and assessed translational differences between and within large and small animals in the CSC therapy field. In addition, we explored the effect of CSC type and other clinically relevant parameters on functional outcome to better predict and design future (pre)clinical studies using CSCs for MI.

Methods

A systematic search was performed, yielding 80 studies. We determined the overall effect of CSC therapy on left ventricular ejection fraction (EF) and performed meta-regression to investigate clinically relevant parameters. We also assessed the quality of included studies and possible bias.

Results

The overall effect observed in CSCs treated animals was 10.7% (95%CI 9.4-12.1 $p<0.001$) improvement in EF compared to placebo controls. Interestingly, CSC therapy had a greater effect in small animals compared to large animals ($p<0.001$). Meta-regression indicated that cell type was a significant predictor for EF improvement in small animals. Minor publication bias was observed in small animal studies.

Conclusion

CSC treatment resulted in significant improvement of EF in preclinical animal models of MI compared to placebo. There was a reduction in the magnitude of effect in large compared to small animal models. Although different CSC types have overlapping culture characteristics, we observed a significant difference in their effect in post-MI animal studies.

Abbreviations

BMMNCs	Bone Marrow Mononuclear Cells
MSCs	Mesenchymal Stem Cells
CSCs	Cardiac Stem Cells
SP	Side Population
Cs	Cardiospheres
CDCs	Cardiosphere Derived Cells
MI	Myocardial Infarction
EF	Ejection Fraction
IS/AAR	Infarct Size per Area at Risk
IS/LV	Infarct Size per Left Ventricle
EDV	End Diastolic Volume
ESV	End Systolic Volume
FS	Fractional Shortening
WT	Wall Thickness
RMD	Raw Mean Difference
SMD	Standardised Mean Difference
95%CI	95% Confidence Interval
NS	Not Significant

Introduction

Cell therapy for ischemic heart disease has entered the arena of clinical trials more than a decade ago.[1,2] Multiple cell types have been used since these first endeavours, all having their pros and cons.[3] There is accumulating evidence that cell types like bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) positively influence the damaged heart through paracrine and/or regenerative mechanisms.[4] The cardiac-derived stem- and progenitor cells have been studied with great interest in this regard, due to their natural location and function in the heart, their proven beneficial effects upon injection after myocardial ischemia and their potential to differentiate towards myocardial lineages.[5] As natural resident cells they could be the ideal candidate for additional therapy after cardiomyocyte loss in both the acute and chronic phases of ischemic cardiac disease. Different types of CSCs have been identified, including the c-kit$^+$, Sca-1$^+$, Islet-1$^+$, the specific side population (SP), cardiospheres (Cs) and cardiosphere derived cells (CDCs), which have all been isolated from the adult heart and characterised to a great extent.[6-11]

CSCs have been extensively studied in animal studies and some are already being applied in clinical trials.[12,13] Some of these CSC types share a highly similar transcriptional program upon culture expansion, suggesting that the reparative mechanisms of these cells on the injured heart might also be, at least partially, similar.[14] Numerous preclinical studies in small and large animals have paved the way for CSCs as a potential therapy for ischemic cardiomyopathies. A meta-analysis of preclinical studies can provide us with critical appraisal of all the current data and add additional insights in potential mechanisms, study design and bias.[15] For CSC treatment in ischemic cardiomyopathy no appraisal and additional analysis exists.

In this meta-analysis, we aimed to quantify the difference between large and small animal studies, provide a comprehensive overview of all studies using CSCs in myocardial infarction (MI) models with functional outcomes and assess different types of bias. Additionally, we aimed to identify key factors that positively influence the outcome after CSC treatment to ultimately provide recommendations for further optimization of design of (pre)clinical stem cell trials.

Methods

For a detailed version of our research protocol we refer to our preregistered and published study protocol.[16] In brief, we performed a search of PubMed and Embase with the search terms "cardiac stem cell", "myocardial infarction" and "animal model" or any of their synonyms. In addition to the search terms defined in our original protocol, we added the terms "cardiac-derived" and cardiosphere-derived" to our original search. Papers were screened by two independent investigators (PPZ, AMDV) in the title-abstract and full-text screen. A third investigator (JS) was consulted in case of no consensus on inclusion. Papers were included if they reported a placebo-controlled MI animal model in which CSCs were administered and in which ejection fraction (EF) as a functional parameter was reported as an outcome. We defined CSCs as cell types which, according to current literature, have been repeatedly shown to reside in the adult heart, have some degree of clonogenicity and have been shown to commit to all cell types of the cardiovascular lineage (Cs, CDCs, c-kit$^+$, Sca-1$^+$, Islet-1$^+$ or SP cells). Importantly, we are interested in the effect of the CSCs only

and therefore exclusively included groups that used non-modified CSCs; we discarded studies that used a scaffold and/or pre-treated CSCs with modifying compounds and/or used CSCs that were genetically modified. In contrast to the information in our protocol, we chose not to pool data for CDCs and Cs in our analyses if we had enough power to separate the two cell types. If too few studies were available, CDCs and Cs were combined.
We used EF as our functional outcome and therefore excluded studies not reporting an EF measurement. If an imaging modality suitable for measuring an EF was used, but authors failed to report this outcome measurement, we contacted the authors. We also consulted authors if crucial data for our analysis was not reported in the paper (number of animals per group, standard errors, etc). Secondary outcomes were infarct size (determined by triphenyl tetrazolium chloride (TTC) staining, Masson's Trichrome staining or MRI, measured for the area at risk (IS/AAR) or for the total left ventricle (IS/LV)), end diastolic volume (EDV), end systolic volume (ESV), infarct wall thickness (WT) and fractional shortening (FS).
We planned to include one variable per 10 included study-groups of comparison, as is commonly accepted for meta-regression.[17] Variables included for meta-regression (in order of interest) were; cell type, study design (immunosuppression), cell characteristics upon administration (autologous/syngeneic/allogeneic/xenogeneic, two-dimensional or three-dimensional culture, cell comorbidity (diseased or healthy)), ischemia model (permanent occlusion or ischemia/reperfusion injury) methodology (CSCs being used as ultimate source or served as controls for empowered CSCs, randomization, blinding (allocation concealment and outcome)). Groups within a variable were included if there were 5 or more comparisons.
All data was inserted into the CAMARADES database and is available upon request.[15]

Statistical analysis

We performed a random effects meta-analysis because of expected heterogeneity, using restricted maximum likelihood. We used raw mean differences (RMDs) for EF, IS/AAR, IS/LV and FS. For EDV, ESV and WT we used standardized mean differences (SMDs), since the (order of) magnitudes of these values and standard errors will differ across species.
If multiple treatment groups with one control group were used in an experiment (e.g. dose finding studies) these counted as multiple comparisons, for which control groups were adjusted to resemble the true effective number of controls per group.[17] For the assessment of potential publication bias we used funnel plots, Egger's regression and Tweedie and Duval's trim and fill analysis.[18,19] Furthermore, we compared the use of CSCs as a control group (for modified CSCs) or being the ultimate treatment being investigated in the experiment. Attrition bias was assessed using the SYRCLE risk of bias tool.[20] Quality of studies was assessed using the CAMARADES checklist.[21]
Exploration of heterogeneity was done using meta-regression. We expected a difference between small and large animals beforehand.[16] After confirming this difference, we used meta-regression to test our variables of interest within the small and large animals. We considered outcomes of the meta-regression to be statistically significant at $p<0.05$ for our primary outcome. If meta-regression was significant, we used a post-hoc Wald test when more than 2 groups were analyzed to explore differences between groups. Additional multivariable meta-regression for all variables of interest was performed with the addition of the variables to the model that significantly explained part of

the heterogeneity. For our secondary outcomes, we applied a Bonferroni-Holmes correction for the three groups we are using (functional, infarct size and wall characteristics), which made the first threshold for significance $p<0.017$.
Data in forest plots and meta-regression analyses is shown as a mean value with 95% confidence interval (95%CI). The post-hoc comparisons on study quality were done using a Mann-Whitney U test when comparing two groups and a Kruskall-Wallis test if more than two groups were used; this data is depicted as mean±SD. Statistical analyses were performed using Stata 11 (Statacorp LP, Texas, USA) and R version 3.1.2 with the additional metafor package.[22]

Results

Our final search was performed on October 29th 2015. We identified 1470 papers on PubMed and 2144 on Embase. After removal of duplicates and title-abstract screening, 345 papers were selected for full-text screening. After our initial search, we included one additional paper, which was identified through reference checking from the search.[23] One study was excluded post-hoc, since it measured functional outcomes directly after CSC therapy. 80 papers were finally included, reporting 1970 animals (1176 treated, 794 controls) giving us 109 comparisons for our primary outcome (Supplementary Figure I, flowchart). All included studies and study characteristics are listed in Supplementary Table I.

Meta-analysis
In an overall estimate of all included studies, CSC treatment culminated in an absolute difference in EF of 10.7% (95%CI 9.4-12.1) compared to control animals (Supplementary Figure II, $p<0.001$). We observed the expected difference in our primary outcome EF between small and large animal studies through meta-regression ($p<0.001$), with subsequent meta-analysis showing an EF difference of 11.7 (95%CI 10.2-13.1, $p<0.001$) for small animals and 5.2 (95%CI 3.4-7.1, $p<0.001$) for large animals (Figure 1A-C). For all secondary outcomes, similar differences for animal size were observed (Supplementary Figure III).

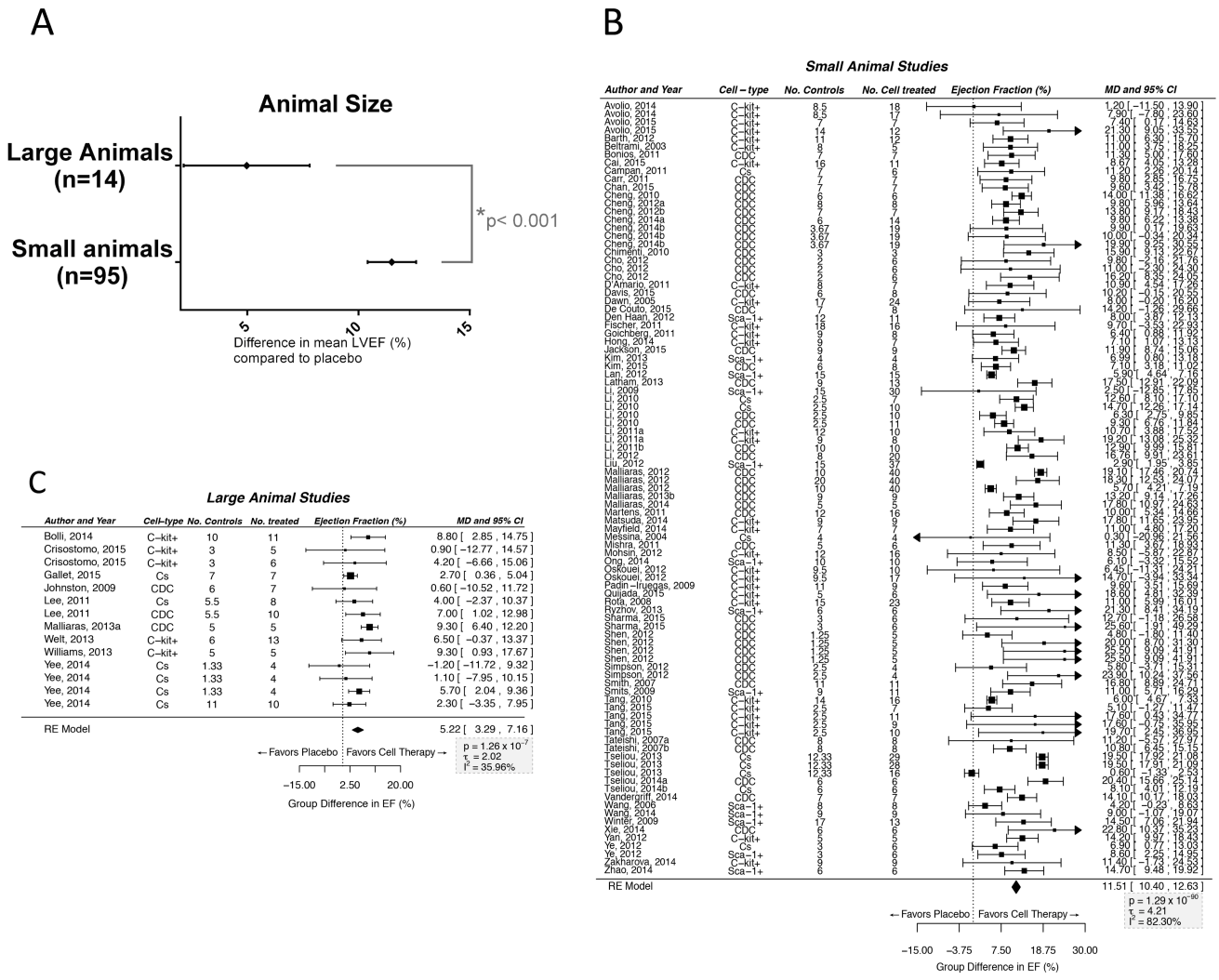

Figure 1. Difference between small and large animal studies. (A) Meta-regression for animal size. (B) Meta-analysis of all included small animal studies. (C) Meta-analysis of all included large animal studies.

Meta-regression

Meta-regression in the small animal studies was used for 10 variables in 95 comparisons. Analyses showed a significant difference in EF for the different cell types, with CDCs appearing more effective compared to only Sca-1$^+$ cells, respectively CDCs: 12.9 (95%CI 11.3-14.5) vs Cs: 11.8 (95%CI 8.7-14.9) vs c-kit$^+$: 10.7 (95%CI 8.5-12.9) vs Sca-1$^+$: 8.3 (95%CI 5.4-11.1), p=0.04 (p=0.006 for post-hoc testing CDCs vs Sca-1$^+$; rest of the comparisons, p=not significant (NS)) (Figure 2A). A descriptive table of all study characteristics per cell type did not show any other variable that coincided with this difference (Supplementary Table II). Immunosuppression, cell source, comorbidity of CSC-donors, culture methods or model of ischemia did not significantly influence outcomes after CSC therapy (Figure 2C-G). Since cell type was a significant predictor, multivariable meta-regression with cell type as an additional co-variable was performed for all variables of interest, in which no differences compared to the initial analyses were seen (meta-regression remained non-significant as in previous univariable analyses). In large animals, the 14 comparisons enabled us to test one variable of interest. In this dataset, Cs/CDCs performed as well as c-kit$^+$ cells (Figure 2B).

For all secondary outcomes (EDV, ESV, IS/AAR, IS/LV, WT, FS), similar trends were observed, all favouring CSC treatment over placebo control in small animal studies (Supplementary Figure IV-IX). For all secondary outcomes, meta-regression was performed, in which cell type and immunosuppression showed an effect on the difference in infarct size (Supplementary Table III). Quality assessment was done for each study (Supplementary Figure XA); post-hoc analyses showed a significant difference in quality between small and large animal studies (Supplementary Figure XB, $p<0.01$). There was no significant difference between different cell types in small animals for these quality parameters (Supplementary Figure XC, p=NS).

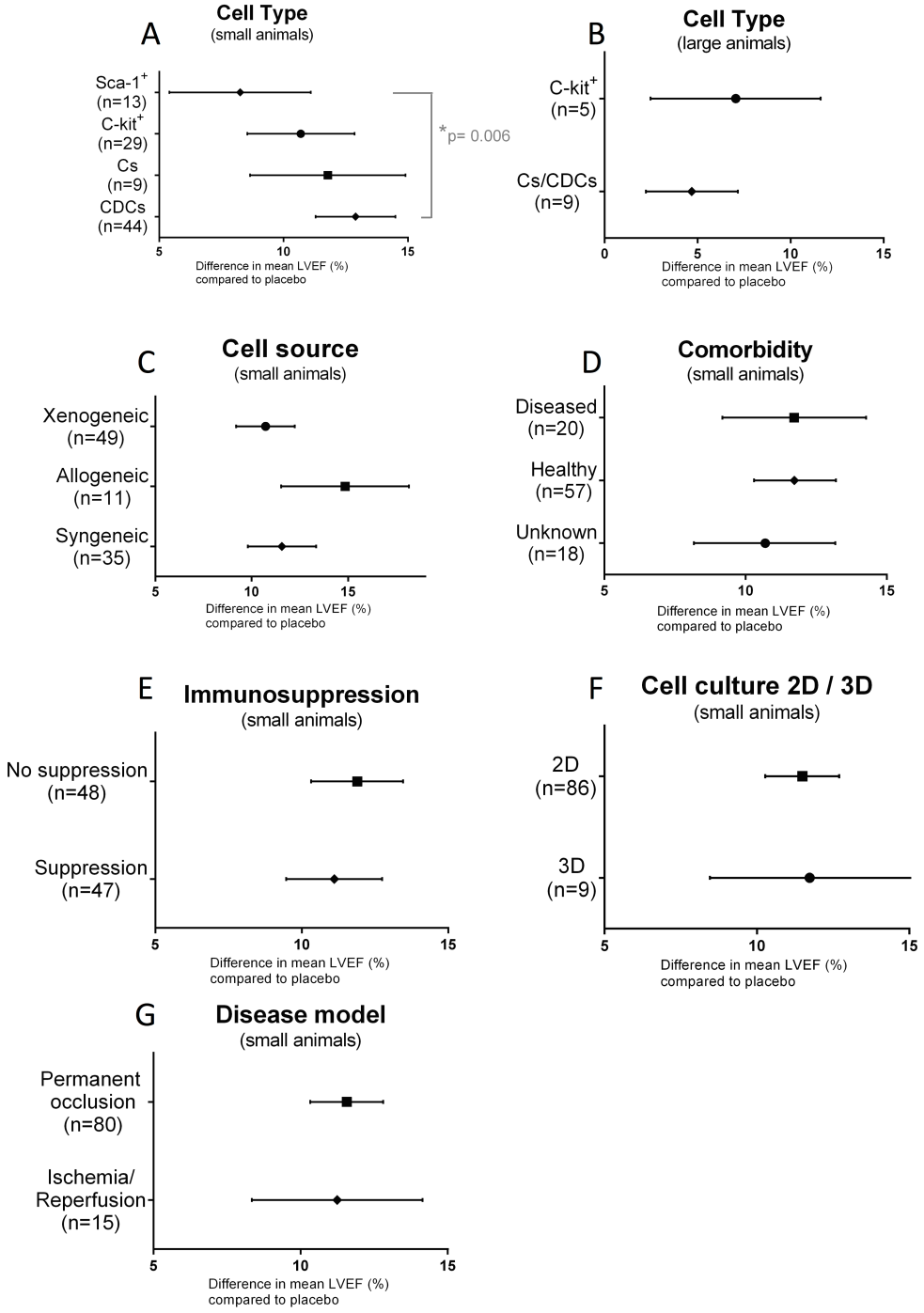

Figure 2. Meta-regression of all variables of interest. (A) Cell type in small animals. (B) Cell type in large animals. (C) Donor source in small animals. (D) Comorbidity of cells in small animals. (E) Immunosuppression in small animals. (F) Cell culture in 2D or 3D in small animals.

Assessment of potential confounding factors

Funnel plot analysis for studies using small animals revealed minor asymmetry for our primary outcome EF (Figure 3A), suggesting potential missing studies. A subsequent trim and fill analysis resulted in the addition of two imputed studies, resulting in a total of 97 comparisons and a negligible reduction in CSC effect size of 0.1% in EF difference (Figure 3A). Egger's regression indicated small study effects in small animal studies (Figure 3B, p= 0.02), again suggesting funnel plot asymmetry. A stratified assessment was done per cell type, again yielding results suggesting publication bias. In trim and fill analyses asymmetric funnel plots with subsequent filling was observed for CDCs, c-kit$^+$ and Sca-1$^+$ cells, accompanied by minor reductions in effect size and significant Egger's regression analyses (Supplementary Figure XI).

The funnel plot for large animal studies showed no asymmetry (Figure 3C), with no studies to be added in the subsequent trim and fill analysis. Egger's regression also did not suggest any small study bias (Figure 3D, p = 0.654).

Meta-regression of the small animal studies showed no significant influence in a direct comparison of the methodological use of randomization (Figure 3E), allocation concealment (Figure 3F) or blinded assessment of the outcome measurement (Figure 3G) on the effect of CSCs on EF. CSCs being used as a control for an empowered treatment performed the same as CSCs being investigated as an ultimate treatment only (Figure 3H). Correcting for the effect of cell type using multivariable meta-regression again did not change these results.

Using the SYRCLE attrition bias tool, 29 studies were classified as low, 46 studies as unknown and 5 studies as a high risk of attrition bias. Interestingly, large animal studies outperformed the small animal studies significantly with regards to the reporting of attrition bias; 8 out of 9 studies were classified as low risk compared to 21 out of 71 studies for the small animal studies. Overall attrition bias, number of enrolled animals and number of drop-outs (if mentioned) was assessed per study (Supplementary Table IV). Small animal studies were more likely to fail to address (potential) exclusion of animals in their experiments compared to large animal studies.

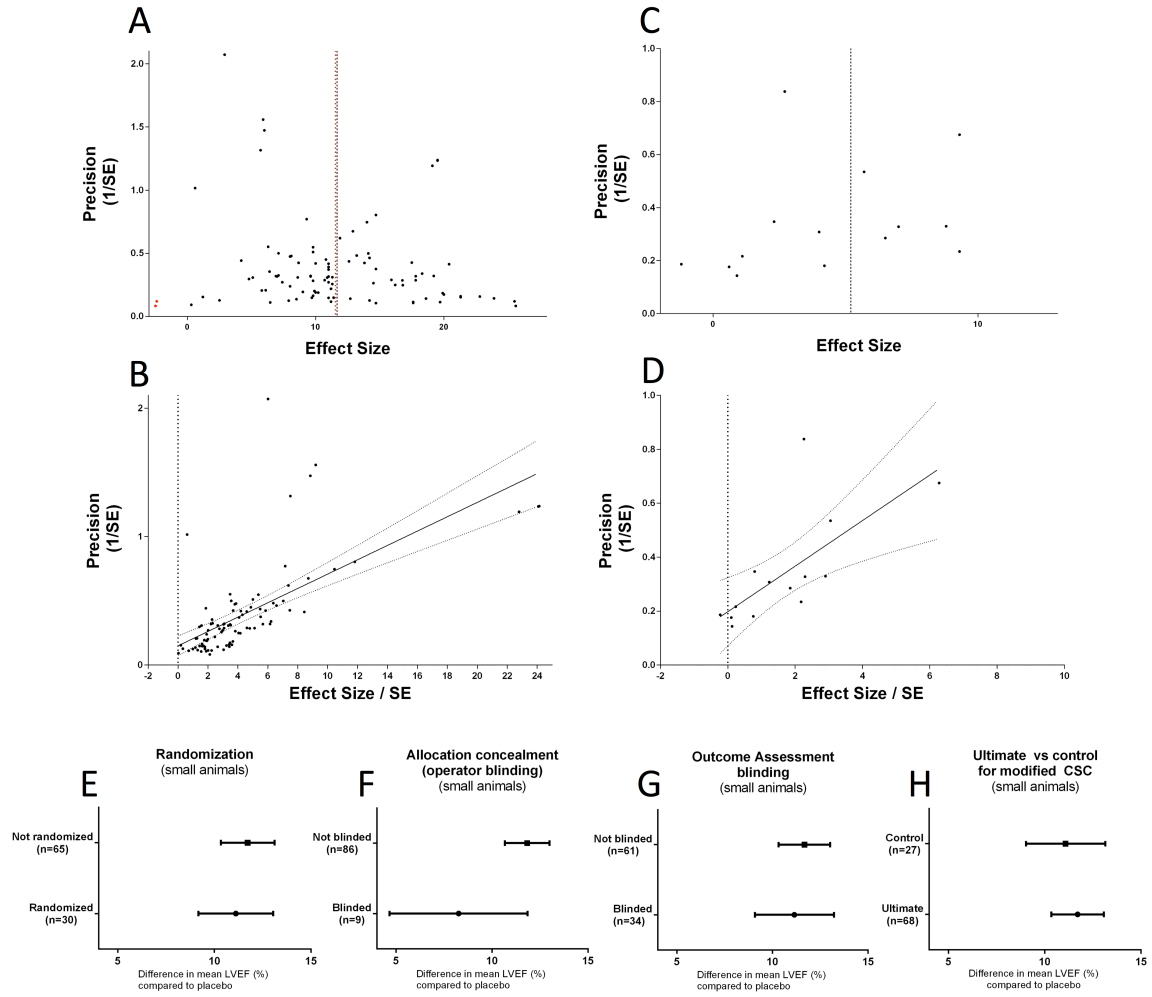

Figure 3. Bias assessment of all included studies. (A) Funnel plot and subsequent trim and fill analysis of small animal studies. Imputed studies and subsequently generated new effect size are depicted in red. (B) Funnel plot of large animal studies. (C) Egger's regression of small animal studies. (D) Egger's regression of large animal studies. Direct comparison for small animal studies was done for (E) allocation concealment, (F) blinding op the operator, (G) blinding of the assessment and (H) the use of CSCs as an ultimate treatment or as a control for an empowered CSC.

Discussion

In this meta-analysis, we assessed the consistent effect of CSC therapy in the infarcted heart. The translational axis, in which effect sizes diminish from small animals to ultimately human studies, is also firmly present in the therapeutic use of CSC therapy for MI. A total of 11 placebo-controlled large animal studies with functional outcome measurements in MI models have been performed to date (Table 1).

The effect of ~5% improvement in EF in large animals upon CSC therapy seems partially maintained at the transition from bench to bedside in RCT's, with the CADUCEUS trial reporting values of ~1% increase in EF over placebo treatment at their 6 month time-point; the clinical reduction in infarct size of ~8% is more optimistic than our data from the large animal studies.[13] The SCIPIO trial showed promising data of ~8% increase in EF after 4 months compared to controls and ~8% reduction in infarct size after CSC treatment compared to baseline values.[24] Since both trials were in small groups without a placebo-control, further research is warranted to confirm these effects.

Study	Animal	No. of controls	No. of treated	Cell type + amount	Donor	Administration timing (after MI)	Assessment timing (after therapy)	Immuno-suppression	EF difference (95%CI)
Bolli, 2013[41]	Pig	10	11	5*10^5 c-kit CSCs	Autologous	5h	20d	No	8.8 (2.9 - 14.7)
Crisostomo, 2015[42]	Pig	6	5	25*10^6 c-kit$^+$ CSCs	Allogeneic	2h	10w	No	4.2 (-6.7 - 15.1)
			6			7d			0.9 (-12.8 -14.6)
Gallet, 2015[43]	Pig	7	7	1.3*10^6 cardiospheres	Allogeneic	3w	4w	No	2.7 (0.4 – 5.04)
Johnston, 2009[44]	Pig	6	7	1*10^7 CDCs	Autologous	4w	8w	No	0.6 (-10.5 - 11.7)
Lee, 2011[45]	Pig	11	10	2*10^6 cardiospheres	Autologous	4w	4w	No	7.0 (1.0 - 13.0)
			8	2*10^6 CDCs					4.0 (-2.4 - 10.4)
Malliaras, 2013[46]	Pig	5	5	12,5*10^6 CDCs	Allogeneic	2-3w	8w	No	9.3 (6.4 - 12.2)
Welt, 2013[47]	Dog	6	13	16*10^5 c-kit$^+$ CSCs	Autologous	6w	24w	No	6.5 (-0.4 - 13.4)
Williams, 2013[28]	Pig	5	5	1*10^6 c-kit$^+$ CSCs	Xenogeneic	2w	4w	Yes	9.3 (0.9 - 17.7)
Yee, 2014[48]	Pig	4	4	15*10^6 cardiospheres	Allogeneic	4w	4w	No	-1.2 (-11.7 - 9.3)
			4	45*10^6 cardiospheres					1.1 (-7.9 - 10.1)
			4	150*10^6 cardiospheres					5.7 (2.0 - 9.4)
		6	6	150*10^6 cardiospheres	Allogeneic	8w	8w	No	2.3 (-3.4 - 8.0)
Jansen of Lorkeers, 2015⌘[30]	Pig	8	8	1*10^7 Sca-1$^+$ CSCs	Xenogeneic	4w	4w	Yes	-1.0 (-9.7-6.7)
Kanazawa, 2015§[49]	Pig	5	4	12.5*10^6 CDCs	Allogeneic	0.5h	2d	No	-

Table 1. Large animal placebo-controlled MI studies using CSCs. ⌘ not included in meta-analysis due to later publication. § not included in meta-analysis due to only short-term functional measurements.

In literature, groups have made head-to-head comparisons between CSCs and other cell types, sometimes claiming superior effects of CSCs compared to MSCs and BMMNCs.[25,26] Although this difference may be present in small animal studies, our pooled effect of large animal CSC studies is actually in the range of the previously pooled effects of MSC and BMMNC therapy (8.0% and 7.6% respectively),[27] and is also in line with a recently conducted direct comparison between c-kit$^+$ CSCs and MSCs in a large animal study.[28] Although this meta-analysis cannot directly compare CSCs with other cell types, its superiority as a cell source to improve global myocardial EF compared to BMMNCs or MSCs is not confirmed by our data and therefore should not be expected in human trials. The combination of for example MSCs and CSCs looks more promising in both small and large animal studies[28,29] and will soon be tested clinically in the recruiting CONCERT-HF trial (NCT02501811).

Our systematic search revealed that only CDCs, Cs, c-kit$^+$ and Sca-1$^+$ cells had multiple studies in which functional measurements were performed, compared to none for the Islet-1$^+$ and SP-cells. There seems to be a difference in the cell type being used and the final effect on functional outcomes in small animal studies, favouring only the CDC over the Sca-1$^+$ group. Any difference in effect between cell types could not be established in large animal studies, which might be partially explained by the reported overlapping transcriptional activity between cultured CSC sub-types.[14]

For this analysis we also had to combine the CDCs and Cs to fully utilize the data. However, due to this combination and the small numbers, this analysis should be interpreted with caution. Furthermore, the Sca-1$^+$ cells were not represented in this analysis. A recent study, which came out later than our systematic search, actually studied the administration of human fetal Sca-1$^+$ cells in a chronic MI model, showing no benefit on functional parameters.[30]
Interest and effort has been put into the donor characteristics and cell availability, deeming either autologous or allogeneic cells superior to one another. In our analyses, we could not find a difference between autologous, allogeneic or even xenogeneic cells, which is also in line with previous findings.[27,31] Despite reports of potential effects of immunosuppression on cell therapy[32] that is often accompanying allogeneic therapy, we could not confirm an effect of immunosuppression on the primary outcome.
Reporting and quality parameters of all trials was widely variable; especially small animal studies extensively differed on quality and failed to report all (secondary) outcomes that could potentially be assessed with the (imaging) techniques available in these studies. The difference in effect size between small and large animals might be partially explained by the more stringent reporting and increase in study quality.

Bias assessments in preclinical CSC studies

Our analyses suggest potential publication bias in small animal studies. Although significant, the publication bias seems minor with only two imputed studies to be added in the overall assessment. Moreover, no major change in effect size after adjustment occurred. However, if we remove the heterogeneity of cell type by doing stratified analyses, the suggested publication bias seems as extensive and present in the majority of the cell types investigated. Although not causing large deviations in effect size, publication bias in preclinical research is an important problem, leading to overall overestimation of effect sizes and efficacy.[33]
Direct comparison of the reporting of randomization, allocation concealment and blinded outcome assessment did not reveal a direct effect, suggesting no bias when not performed. Nonetheless, we strongly recommend implementing these in standard methodology, since it is commonly known that these measures effectively protect against overestimation of the effect and lead to bias reduction.[34] Lastly, there was a risk of attrition bias in a number of studies in especially the small animal data. This might influence outcomes too; a recent report showed that this also can lead to overestimation of effect sizes.[35]

Heterogeneity is a key condition for the execution of meta-regression in animal studies, but it can also cause confusion if heterogeneity is not well balanced. Because of this, we did additional multivariable regression to correct for the cell type effect and an additional stratified summary of our results on cell type to investigate the impact of differences in methodology. We also observed selective reporting of outcomes, particularly in small animal studies on outcomes; this might call for more standard reporting guidelines for animal studies in cardiac regeneration.
For comparability, we only included individual CSC treatment and therefore excluded all studies using modified CSCs and/or CSCs in combination with biomaterials, which is a possible limitation of this study. For CSC transplantation therapy, several biomaterials and enhancing proteins show great promise in boosting the effects of CSC therapy, enhancing effects that we cannot quantify in

our analyses due to their heterogeneity and small sample size. Nonetheless, it is possible that biomaterials[36], growth factors (e.g. IGF/HGF)[37,38], and other compounds or proteins like necrostatin[39] and pim-1[40] can boost retention, survival, biological function and ultimately the regenerative effects of cell therapy. Furthermore, we excluded all studies that did not report an EF measurement; hence, studies reporting only our secondary outcomes were not included in our analysis. Although strict, we think reporting EF measurements also serves as an extra quality control in this study. Besides exclusion of studies, relevant studies could still be missed by our systematic approach; a clear example is the study we found through the screening of references, while the actual paper was not identified using the search terms in our primary search.

In summary, we have provided a first-ever comprehensive systematic overview of therapeutic placebo-controlled CSC animal studies and quantified the difference in effect along the translational axis. Furthermore, we discovered key differences and similarities in these studies in both small and large animal studies through meta-regression. The compiling preclinical evidence supports the use of these CSCs in multiple ischemic cardiomyopathy scenarios, with both allogeneic and autologous cells as an option. The next few years will be interesting as clinical trials using CSCs will hopefully give us more answers on the transition of CSC therapy in clinical care. That being said, the superiority of CSCs compared to other (more easily accessible) cell types has to be proven in the clinical setting to make it an acceptable alternative.

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Novelty and Significance

What is known?

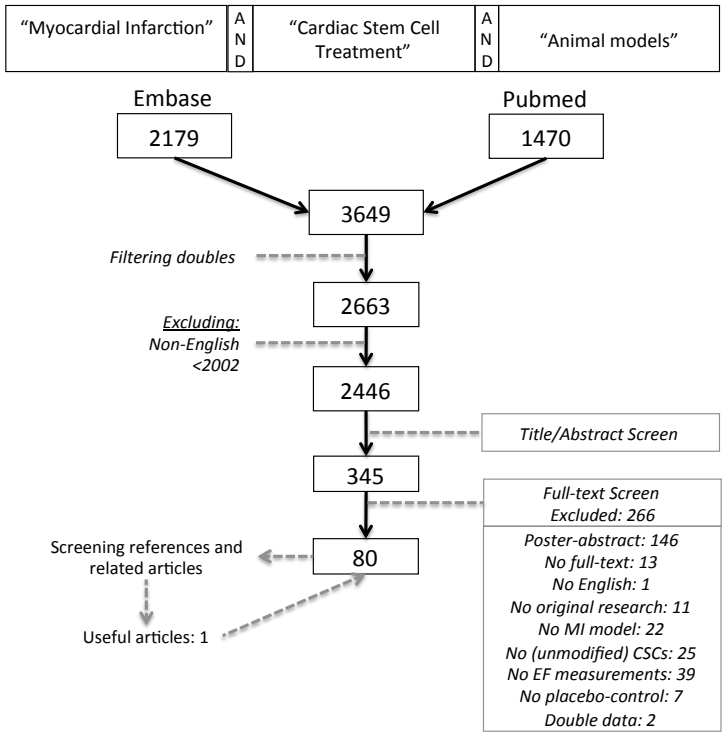
- Cardiac stem cells (CSCs) have been found to reside in the adult heart and can differentiate towards cell types of the cardiovascular lineages
- CSCs have shown great potential as a regenerative therapeutic upon myocardial infarction (MI) in animal models and are currently being tested in clinical studies

What new information does this article contribute?

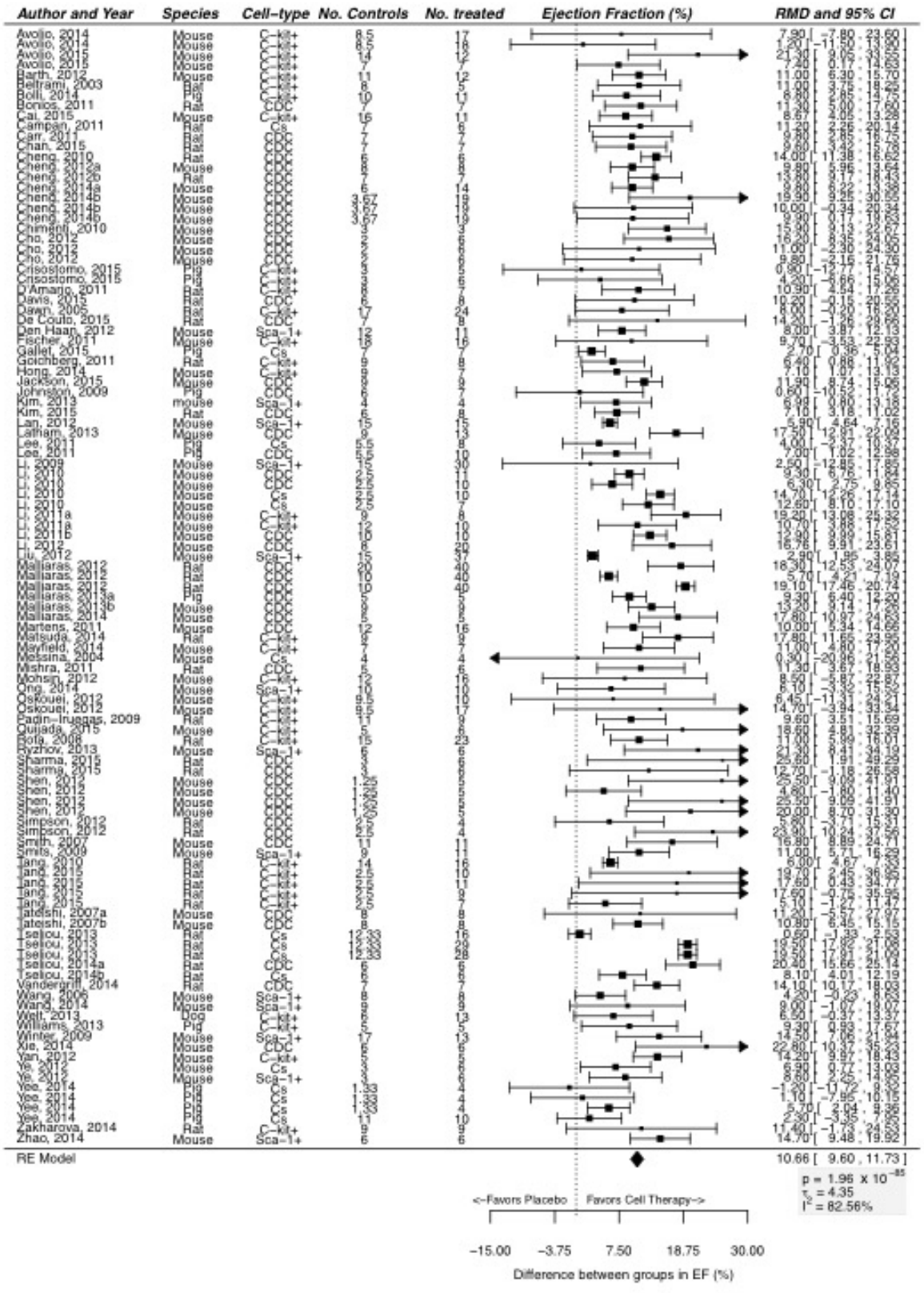
- A consistent therapeutic effect of CSC therapy on cardiac function after MI is systematically shown through meta-analysis
- CSC therapy was associated with a ~12% increase in ejection fraction in small animals, compared to a ~5% increase in ejection fraction in large animal models of MI
- There is a difference between small and large animal models, not only in effect size but also in study quality and attrition bias

Cardiac stem cell (CSC) therapy is suggested as one of the most promising cell types used for cell therapy after MI. No systematic overview and subsequent meta-analysis of preclinical data exists to date. Our systematic approach, yielding 80 studies and including 1970 animals, confirms the consistent effect of CSCs and provides us with a first comprehensive overview of pre-clinical MI studies in an unbiased and systematic manner. From our meta-analysis, it is evident that CSC therapy has more beneficial effects in small animal models than in large animals. Our analyses suggest that some types of CSCs might be more beneficial than others. Furthermore, there seems to be minor publication bias in the field of CSC therapy, which apparently has limited influence on the reported effects. This new information gives us an extra confirmation of the effect of CSCs in preclinical studies, thereby suggesting no influence of immunosuppression, cell source, comorbidity of CSC-donors, culture methods or model of ischemia. Moreover, it also confirms the need for appropriate testing and quality controls in both small and large animal models of MI for adequate translation to clinical studies.

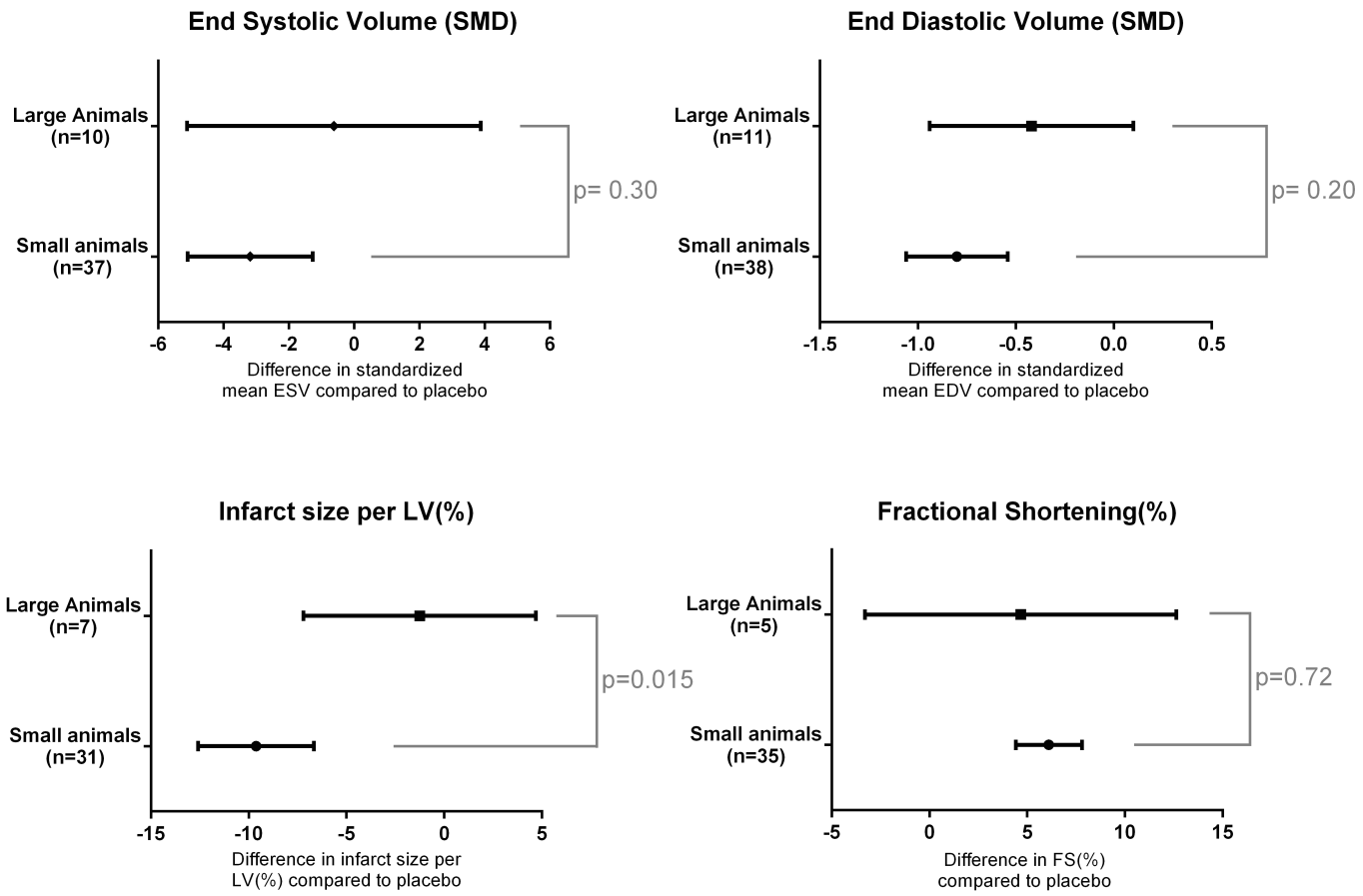
Supplementary Figures & Tables



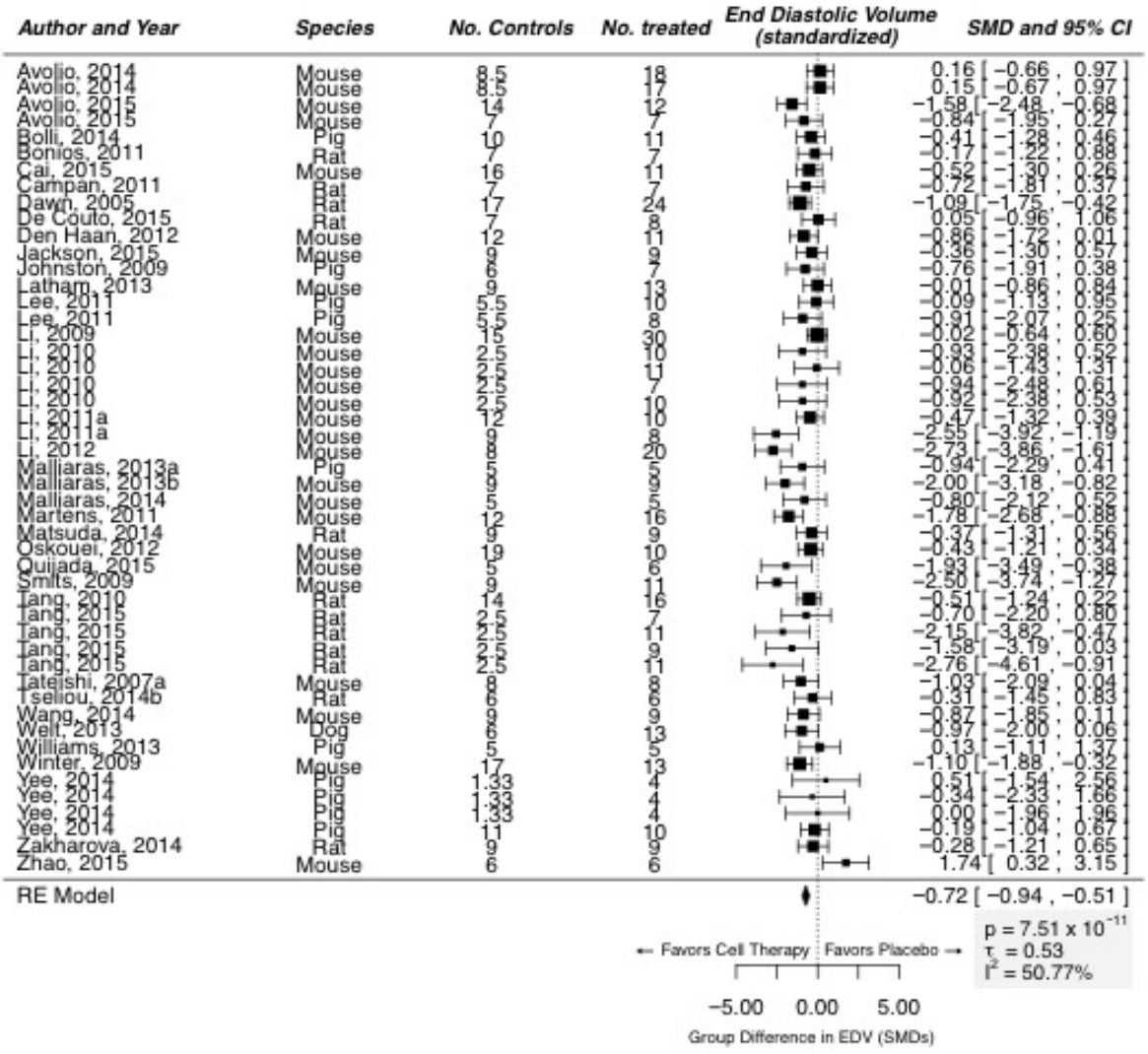
Supplementary Figure I. Flowchart of the systematic search, conducted on October 29th 2015.



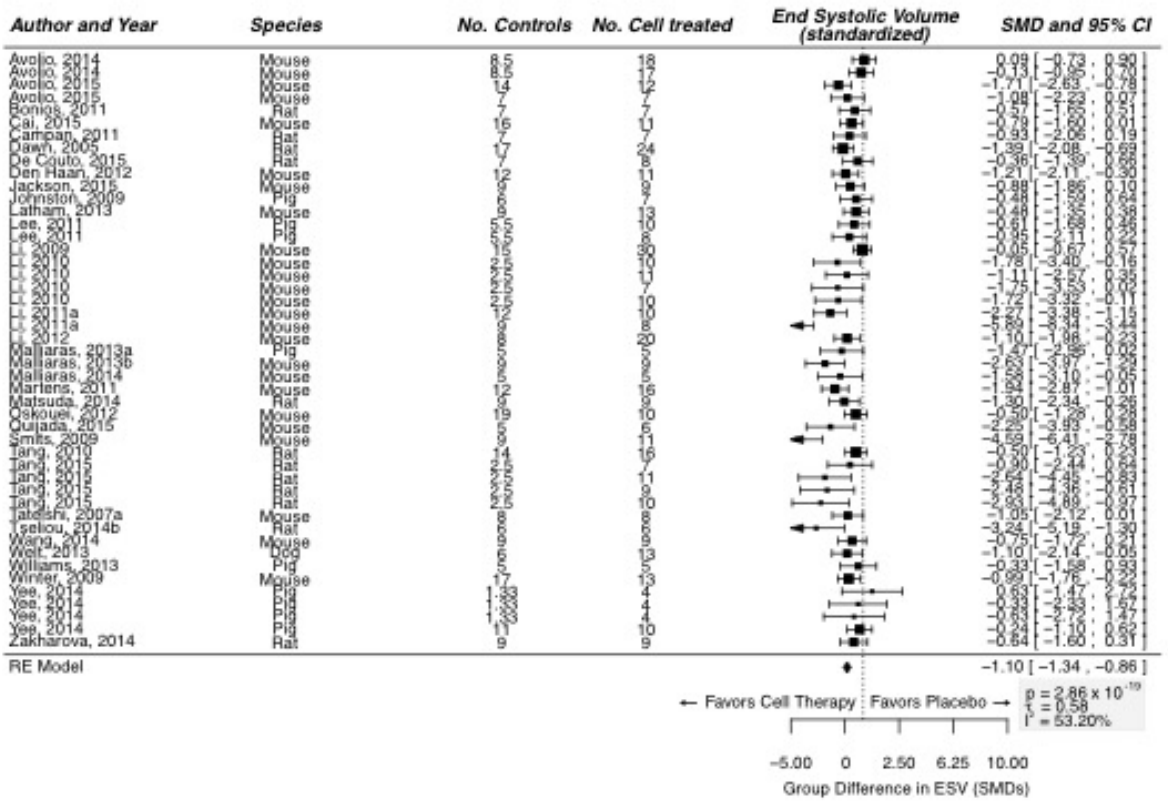
Supplementary Figure II. Meta-analysis of all included studies on the raw mean difference of ejection fraction between CSC treatment and placebo.



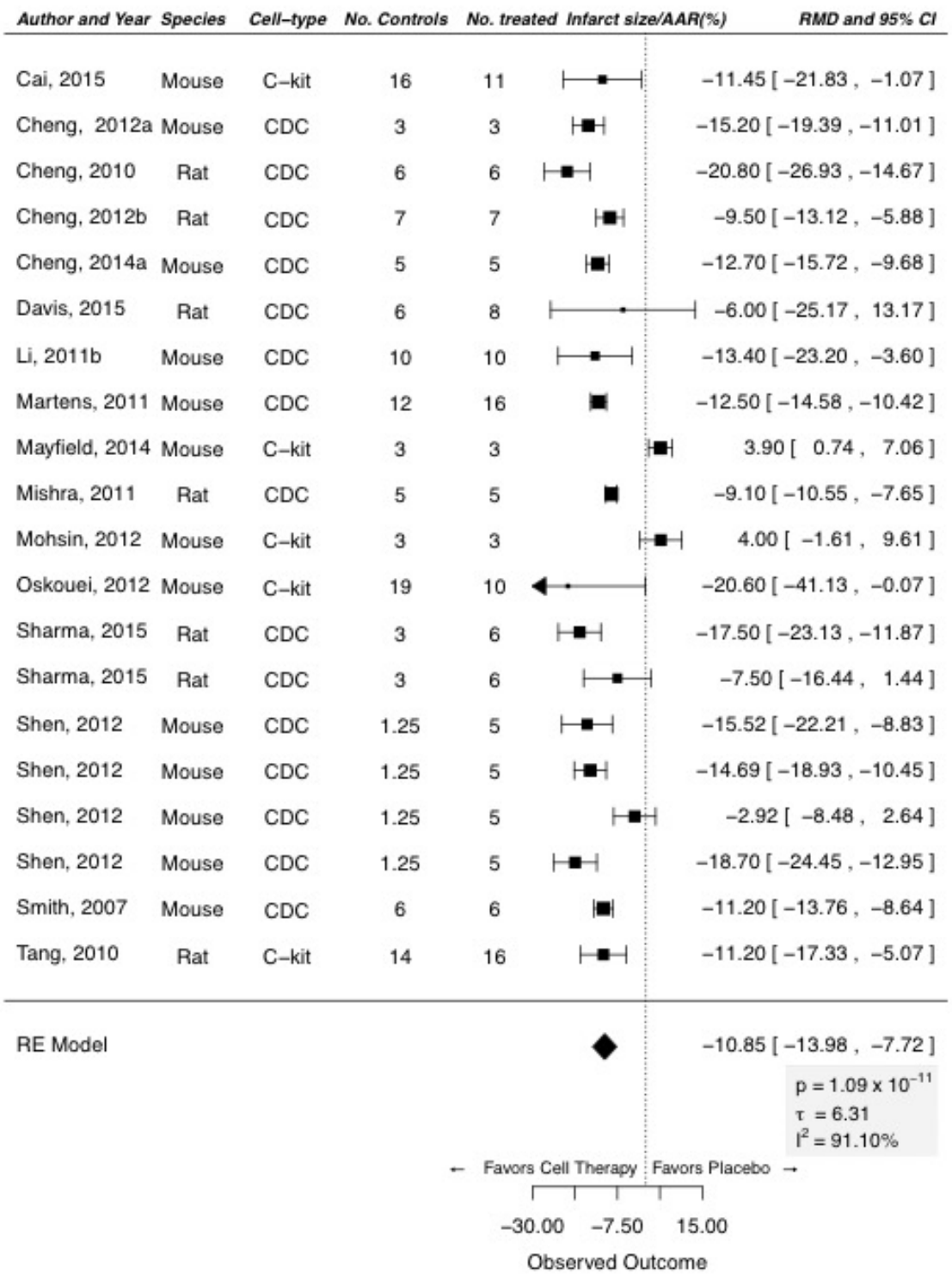
Supplementary Figure III. Meta-regression of all secondary outcomes on the difference between large and small animal studies. For ESV and EDV a standardized mean difference (SMD) is used, while for FS and IS/LV a raw mean difference is depicted for the difference between CSC treatment and placebo. For IS/AAR and WT not enough comparisons were available to do meta-regression.



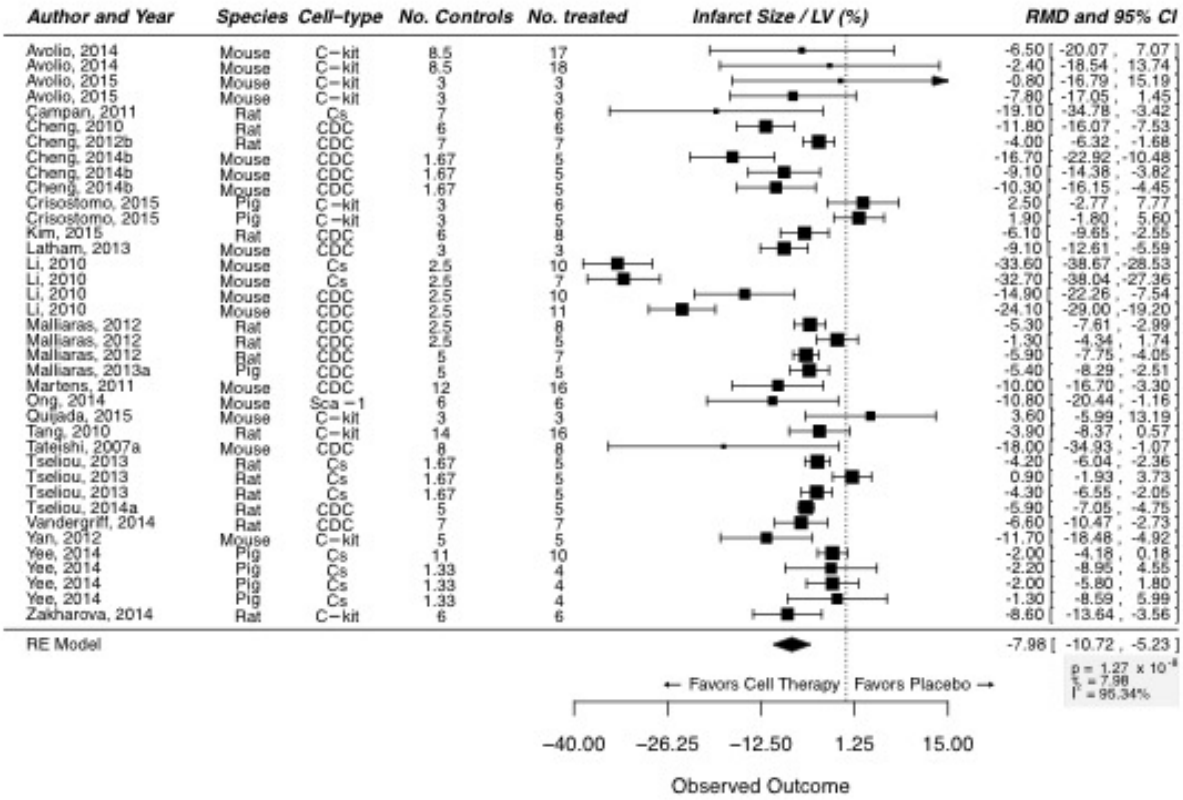
Supplementary Figure IV. Meta-analysis of all included studies on the standardized mean difference of end diastolic volume between CSC treatment and placebo.



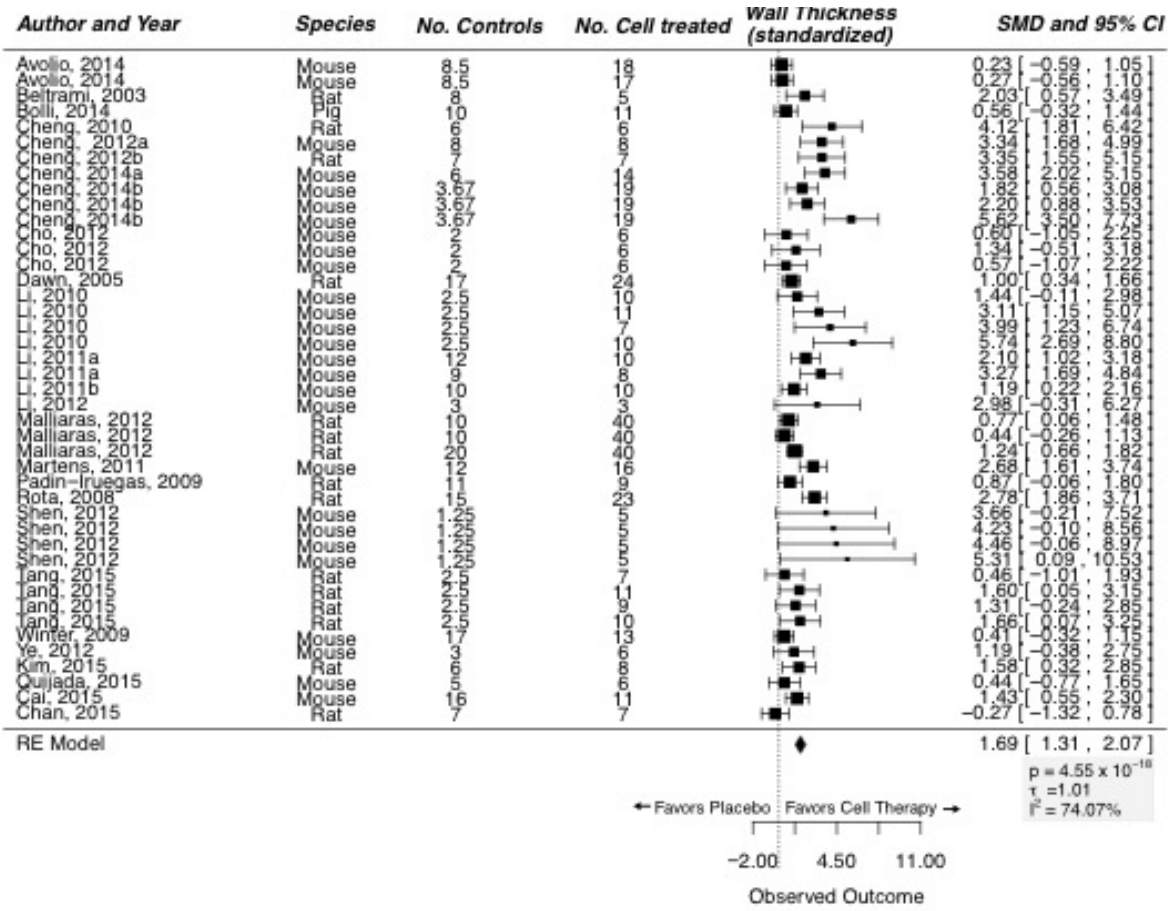
Supplementary Figure V. Meta-analysis of all included studies on the standardized mean difference of end systolic volume between CSC treatment and placebo.



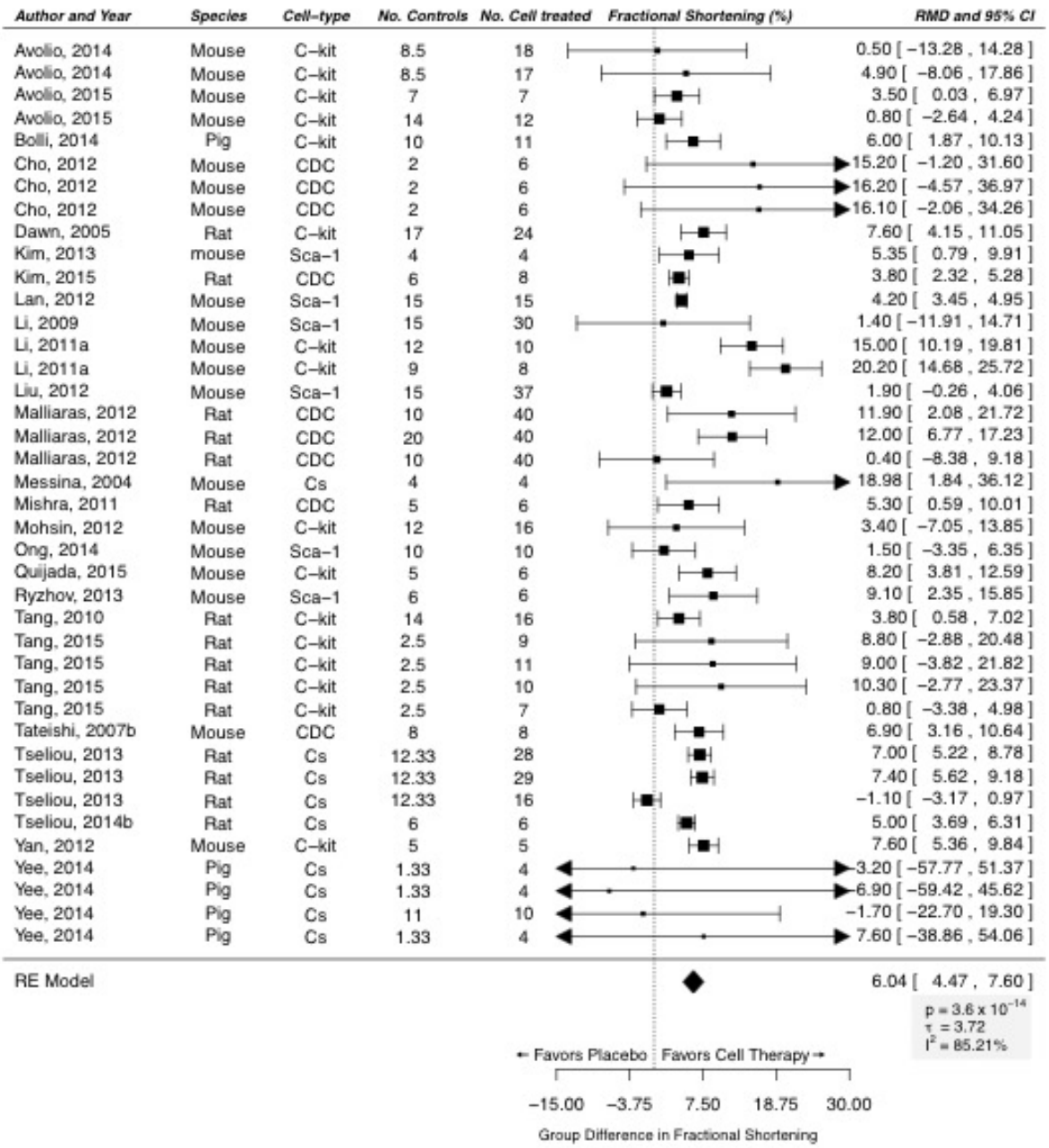
Supplementary Figure VI. Meta-analysis of all included studies on the raw mean difference of infarct size of the area at risk (IS/AAR) between CSC treatment and placebo.



Supplementary Figure VII. Meta-analysis of all included studies on the raw mean difference of infarct size of the left ventricle (IS/LV) between CSC treatment and placebo.



Supplementary Figure VIII. Meta-analysis of all included studies on the standardized mean difference of wall thickness between CSC treatment and placebo.

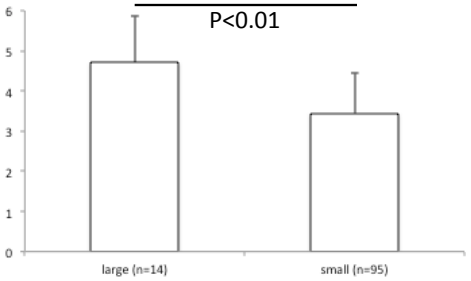


Supplementary Figure IX. Meta-analysis of all included studies on the raw mean difference of fractional shortening between CSC treatment and placebo.

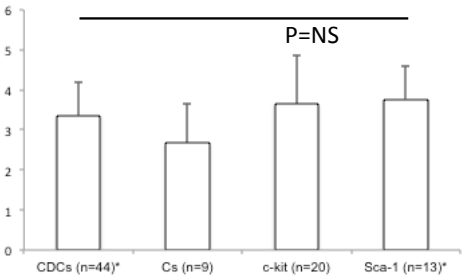
A

Author	Cell type	Animal	Peer Review Publication	Control of Temperature	Random Allocation to Group	Blinded Induction of Ischaemia	Blinded Assessment of Outcome	Sample Size Calculation	Compliance with Animal Welfare Regulations	Conflict of Interest disclosed	TOTAL
Avolio, 2014	C-kit+	small	+	-	-	-	-	-	+	+	3
Avolio, 2014	C-kit+	small	+	-	-	-	-	-	+	+	3
Avolio, 2015	C-kit+	small	+	-	-	-	-	-	+	+	3
Avolio, 2015	C-kit+	small	+	-	-	-	-	-	+	+	3
Barth, 2012	C-kit+	small	+	-	-	-	-	-	-	+	2
Beltrami, 2003	C-kit+	small	+	-	-	-	-	-	+	-	2
Bolli, 2014	C-kit+	large	+	+	-	-	-	-	+	-	3
Bonios, 2011	CDC	small	+	-	+	-	-	-	+	-	3
Cai, 2015	C-kit+	small	+	+	-	-	-	+	+	+	5
Campan, 2011	Cs	small	+	-	+	-	-	-	+	+	4
Carr, 2011	CDC	small	+	-	-	-	-	-	+	+	3
Chan, 2015	CDC	small	+	+	-	-	-	-	-	-	2
Cheng, 2010	CDC	small	+	-	-	-	-	-	+	+	3
Cheng, 2012a	CDC	small	+	-	-	-	-	-	-	+	3
Cheng, 2012b	CDC	small	+	-	-	-	-	-	+	+	3
Cheng, 2014a	CDC	small	+	-	-	-	+	-	+	+	4
Cheng, 2014b	CDC	small	+	-	-	-	+	-	+	+	4
Cheng, 2014b	CDC	small	+	-	-	-	+	-	-	+	3
Cheng, 2014b	CDC	small	+	-	-	-	+	-	-	+	3
Chimenti, 2010	CDC	small	+	-	-	-	-	-	-	+	2
Cho, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Cho, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Cho, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Crisostomo, 2015	C-kit+	large	+	-	-	-	+	-	+	+	4
Crisostomo, 2015	C-kit+	large	+	-	-	-	+	-	+	+	4
D'Amario, 2011	C-kit+	small	+	-	-	-	-	-	-	+	2
Davis, 2015	CDC	small	+	-	-	-	+	-	-	+	3
Dawn, 2005	C-kit+	small	+	+	-	-	-	-	-	-	2
De Couto, 2015	CDC	small	+	-	+	-	-	-	+	+	4
Den Haan, 2012	Sca-1+	small	+	-	-	-	-	-	+	+	3
Fischer, 2011	C-kit+	small	+	-	-	+	-	-	+	+	4
Gallet, 2015	Cs	large	+	-	+	+	+	-	+	+	6
Goichberg, 2011	C-kit+	small	+	+	-	-	-	-	+	+	4
Hong, 2014	C-kit+	small	+	+	-	-	-	-	+	+	4
Jackson, 2015	CDC	small	+	-	-	-	-	-	+	+	4
Johnston, 2009	CDC	large	+	-	+	-	-	-	+	+	4
Kim, 2013	Sca-1+	small	+	-	-	+	+	-	+	+	5
Kim, 2015	CDC	small	+	-	+	-	-	-	+	-	4
Lan, 2012	Sca-1+	small	+	-	+	-	+	-	-	+	5
Latham, 2013	CDC	small	+	-	-	-	+	-	-	+	3
Lee, 2011	CDC	large	+	-	+	+	-	-	-	+	4
Lee, 2011	Cs	large	+	-	+	+	-	-	-	+	4
Li, 2009	Sca-1+	small	+	-	-	-	+	-	+	-	3
Li, 2010	CDC	small	+	-	+	-	-	-	-	+	3
Li, 2010	CDC	small	+	-	+	-	-	-	-	+	3
Li, 2010	Cs	small	+	-	+	-	-	-	-	+	3
Li, 2010	Cs	small	+	-	+	-	-	-	-	+	3
Li, 2011a	C-kit+	small	+	+	+	-	+	-	+	-	5
Li, 2011a	C-kit+	small	+	+	+	-	+	-	+	-	5
Li, 2011b	CDC	small	+	-	+	-	-	-	+	+	4
Li, 2012	CDC	small	+	-	-	-	+	-	+	+	3
Liu, 2012	Sca-1+	small	+	-	+	+	+	-	-	+	5
Malliaras, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Malliaras, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Malliaras, 2012	CDC	small	+	-	-	-	-	-	+	+	3
Malliaras, 2013a	CDC	large	+	-	+	-	+	-	+	+	5
Malliaras, 2013b	CDC	small	+	-	+	-	-	-	+	+	4
Malliaras, 2014	CDC	small	+	-	-	-	-	-	+	+	3
Martens, 2011	CDC	small	+	-	-	-	-	-	+	+	3
Matsuda, 2014	C-kit+	small	+	-	-	-	-	-	-	-	2
Mayfield, 2014	C-kit+	small	+	-	-	-	-	-	-	+	2
Messina, 2004	Cs	small	+	-	-	-	-	-	-	-	1
Mishra, 2011	CDC	small	+	+	+	-	+	-	-	+	5
Mohsin, 2012	C-kit+	small	+	-	-	-	-	-	+	+	3
Ong, 2014	Sca-1+	small	+	-	+	-	-	-	+	+	4
Oskouei, 2012	C-kit+	small	+	+	-	-	+	-	+	+	5
Oskouei, 2012	C-kit+	small	+	+	-	-	+	-	+	+	5
Padin-Iruegas, 2009	C-kit+	small	+	-	-	-	-	-	-	+	2
Quijada, 2015	C-kit+	small	+	-	-	-	+	-	+	+	4
Rota, 2008	C-kit+	small	+	-	-	-	+	-	-	+	3
Ryzhov, 2013	Sca-1+	small	+	-	-	-	+	-	+	-	3
Sharma, 2015	CDC	small	+	-	+	+	+	-	-	+	5
Sharma, 2015	CDC	small	+	-	+	+	+	-	-	+	5
Shen, 2012	CDC	small	+	-	+	-	-	-	-	+	3
Shen, 2012	CDC	small	+	-	+	-	-	-	-	+	3
Shen, 2012	CDC	small	+	-	+	-	-	-	-	+	3
Shen, 2012	CDC	small	+	-	+	-	-	-	-	+	3
Simpson, 2012	CDC	small	+	-	-	-	+	-	+	+	5
Simpson, 2012	CDC	small	+	-	-	-	+	-	+	+	5
Smith, 2007	CDC	small	+	-	-	-	-	-	+	+	3
Smits, 2009	Sca-1+	small	+	-	-	+	+	-	-	+	4
Tang, 2010	C-kit+	small	+	+	+	-	-	-	-	+	4
Tang, 2015	C-kit+	small	+	+	-	-	+	-	+	+	5
Tang, 2015	C-kit+	small	+	+	-	-	+	-	+	+	5
Tang, 2015	C-kit+	small	+	+	-	-	+	-	+	+	5
Tang, 2015	C-kit+	small	+	+	-	-	+	-	+	+	5
Tateishi, 2007a	CDC	small	+	-	-	-	-	-	+	+	3
Tateishi, 2007b	CDC	small	+	-	-	-	-	-	+	+	3
Tseliou, 2013	Cs	small	+	-	-	-	-	-	-	+	2
Tseliou, 2013	Cs	small	+	-	-	-	-	-	-	+	2
Tseliou, 2013	Cs	small	+	-	-	-	-	-	-	+	2
Tseliou, 2014a	CDC	small	+	-	-	-	-	-	+	+	3
Tseliou, 2014b	Cs	small	+	-	+	-	-	-	-	+	3
Vandergriff, 2014	CDC	small	+	-	+	-	-	-	+	+	4
Wang, 2006	Sca-1+	small	+	-	-	-	-	-	+	+	3
Wang, 2014	Sca-1+	small	+	-	-	-	-	-	+	+	3
Welt, 2013	C-kit+	large	+	-	+	-	-	-	+	+	5
Williams, 2013	C-kit+	large	+	-	-	-	-	-	+	+	3
Winter, 2009	Sca-1+	small	+	-	+	-	-	-	+	+	4
Xie, 2014	CDC	small	+	-	+	+	+	-	-	+	5
Yan, 2012	C-kit+	small	+	-	+	-	-	-	+	+	4
Ye, 2012	Cs	small	+	-	-	-	+	-	+	+	4
Ye, 2012	Sca-1+	small	+	-	-	-	+	-	+	+	4
Yee, 2014	Cs	large	+	-	+	+	+	-	+	+	6
Yee, 2014	Cs	large	+	-	+	+	+	-	+	+	6
Yee, 2014	Cs	large	+	-	+	+	+	-	+	+	6
Yee, 2014	Cs	large	+	-	+	+	+	-	+	+	6
Zakharova, 2014	C-kit+	small	+	-	+	-	-	-	+	+	5
Zhao, 2014	Sca-1+	small	+	-	-	-	-	-	+	+	3

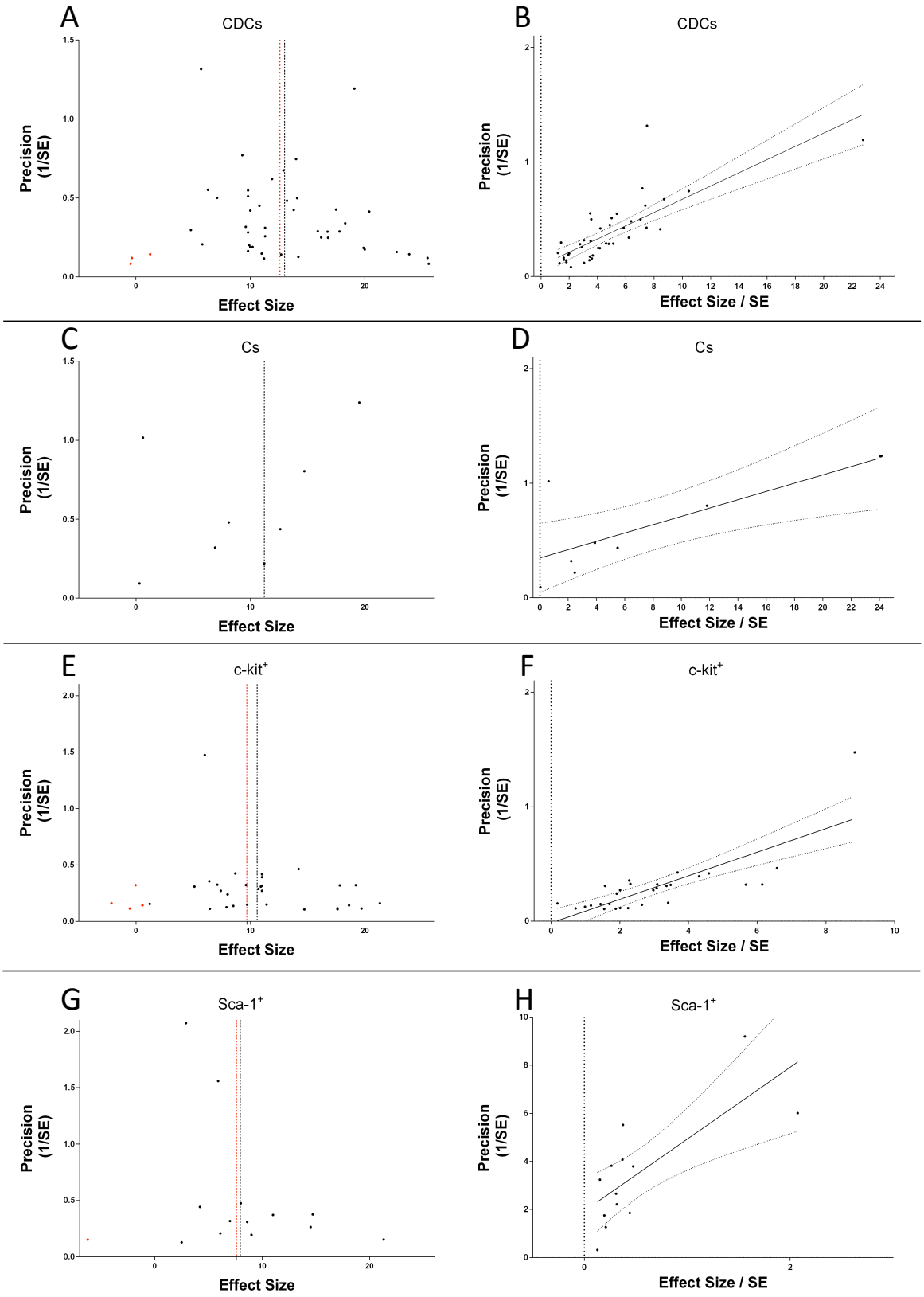
B



C



Supplementary Figure X. Study quality measurements. (A) Quality measurements per study based on the CAMARADES checklist. (B) Difference between large and small animals in quality, using the Mann-Whitney U test. (C) Difference between cell type, using the Kruskall-Wallis test. *Ye et al. is counted twice for the cell type comparison in the small animal studies, since they used both Sca-1+ cells as CDCs.



Supplementary Figure XI. Bias assessment of all included studies per cell type using funnel plots with subsequent trim and fill (imputed studies and subsequently generated new effect size are depicted in red) and Egger's regression. (A) CDC Funnel plot and subsequent trim and fill analysis (4 studies to be added). (B) CDC Egger's regression, p=0.20. (C) Cs Funnel plot without small study effect. (D) Cs Egger's regression, p=0.451. (E) c-kit+ Funnel plot and subsequent trim and fill analysis (4 studies to be added). (F) c-kit+ Egger's regression, p=0.001. (G) sca-1+ Funnel plot and subsequent trim and fill analysis (1 study to be added). (H) sca-1+ Egger's regression, p=0.009.

First author	Animal model	Sex	Age	Type of injury	Cell type	Cell source species	Autologous / Allogeneic / Xenogeneic	Amount of cells	Administration	Immunosuppr.	Control	Time of cell adm.	Measure time after MI	Measure time after cell injection
Avolio, 2014 [1]	SCID-Beige mice	F	8w	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	3*10^5	Injected in border zone	Y	Vehicle	Immediately after MI	2w	2w
Avolio, 2015 [2]	SCID-Beige mice	F	8w	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	3*10^5	Injected in border zone	Y	PBS	Immediately after MI	14d	14d
Barth, 2012 [3]	SCID mice	M	10-20w	Permanent LAD ligation	c-kit+ CSC	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Beltrami, 2003 [4]	Fischer 344 rats	F	2m	Permanent LAD ligation	c-kit+ CSCs	Rat	Syngeneic	2*10^5	Injected in border zone	N	PBS	5h after MI	10d, 20d	10d, 20d
Bolli, 2013 [5]	Yorkshire pigs	M	8-10w	90-min LAD ligation	c-kit+ CSCs	Porcine	Autologous	5*10^5	Intracoronary infusion	N	Vehicle	3-4 months after MI	4-5 months	1m
Bonios, 2011 [6]	WKY rats	F	3m	Permanent LAD ligation	CDCs	Rat	Syngeneic	2*10^6	Injected in infarct regions	N	PBS	Immediately after MI	1w, 4w	1w, 4w
Cai, 2015 [7]	SCID mice	M	11-12w	45-min balloon occlusion around mid-LAD	c-kit+ CSCs	Human	Xenogeneic	5*10^5	Injected into borderzone	Y	Vehicle	30min after MI	5w	5w
Campan, 2011 [8]	Wistar rats	M	adult	Permanent LAD ligation	Cs	Pig	Xenogeneic	1*10^6	Injected intro border zone	N	PBS	45 minutes after MI	6w	6w
Carr, 2011 [9]	SD rats	F		50-min LAD ligation	CDCs	Rat	Allogeneic	Total of 6*10^6 (in two days)	Injected in border zone and tail vein	N	Medium	10min after reperfusion	2w, 6w, 10w, 16w	2w, 6w, 10w, 16w
Chan, 2015 [10]	WKY rats	F	10-12w	Permanent LAD ligation	CDCs	Rat	Syngeneic	1*10^6	Intramyocardial injection	N	Medium	Immediately after MI	4w	4w
Cheng, 2010 [11]	WKY rats	F		Permanent LAD ligation	CDCs	Rat	Syngeneic	1*10^6	Injected in border zone	N	PBS	Immediately after MI	3w	3w
Cheng, 2012a [12]	SCID-Beige mice	M	10-20w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1,5*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Cheng, 2012b [13]	WKY rats	F		45-min LAD ligation	CDCs	Rat	Syngeneic	5*10^5	Injected into the left ventricle cavity	N	PBS	20m after reperfusion	3w	3w
Cheng, 2014a [14]	SCID-Beige mice	M	10-12w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Cheng, 2014b [15]	SCID mice	M	10-12w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Chimenti, 2010 [16]	SCID mice		10w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	Fibroblasts	Immediately after MI	1w, 3w	1w, 3w
Cho, 2012 [17]	Balb/c mice	Both		Permanent LAD ligation	CDCs	Mouse	Allogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	2w, 10w	2w, 10w
Crisostomo, 2015 [18]	Domestic swine	F		90-min LAD balloon occlusion	c-kit+ CSCs	Pig	Allogeneic	25*10^6	Intracoronary injection	N	PBS	2h, 7d after MI	10w	9w, 10w
D'amario, 2011 [19]	Fischer 344 rats	F	3m	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	10d	10d
Davis, 2010 [20]	WKY rats		8-9w	Permanent LAD ligation	CDCs	Rat	Syngeneic	1*10^6	Injected in border zone	N	PBS	Immediately after MI	3w, 6w	3w, 6w
Dawn, 2005 [21]	Fischer 344 rats	F	3m	90-min LAD ligation	c-kit+ CSCs	Rat	Syngeneic	1*10^6	Infusion into aortic root	N	PBS	4h after reperfusion	5w	5w
De Couto, 2015 [22]	WKY rats	F	7-10w	45-min LAD ligation, 20-min reperfusion, then permanent LAD ligation	CDCs	Rat	Allogeneic	5*10^5 after IR + 2*10^6 after MI	Injected in border zone	N	PBS	20 mins after MI	2w	2w
den Haan, 2012 [23]	NOD-SCID mice	M	8-10w	Permanent LAD ligation	Sca-1+ CSCs	Human	Xenogeneic	2*10^5	Injected in border zone	Y	Medium	20min after MI	2w	2w
Fischer, 2011 [24]	FVB mice	F	10-12w	Permanent LAD ligation	c-kit+ CSCs	Mice	Syngeneic	1*10^5	Injected in border zone	N	PBS	5min after ligation	0-12weeks	0-12weeks
Gallet, 2015 [25]	Yucatan minipigs	F	adult	2.5h LAD balloon occlusion	Cs	Pig	Allogeneic	50*10^6	Intracoronary infusion	N	Vehicle	3w after MI	7-8w	4w
Goichberg, 2011 [26]	Fisher 344 rats	F	3m	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	5*10^4	Injected in border zone	Y	PBS	Immediately after MI	2w	2w
Hong, 2014 [27]	C57BL6/J mice	F	11-12w	60-min LAD ligation	c-kit+ CSCs	Mouse	Syngeneic	1*10^5	Intracoronary infusion	N	PBS	48h after MI	37d	5w
Jackson, 2015 [28]	NOD-SCID mice		6w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injections between apex and border zone	Y	PBS	1w after MI	4w	3w
Johnston, 2009 [29]	Minipigs			2.5h LAD balloon occlusion	CDCs	Pig	Autologous	1*10^7	Intracoronary infusion	N	Vehicle	4 weeks after MI	12w	8w
Kim, 2013 [30]	C57BL6/J mice	F	12 weeks	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngeneic	2*10^5	Injected in border zone	N	PBS	Immediately after MI	4d, 7d, 28d	4d, 7d, 28d

Kim, 2015 [31]	SD rats	M	10w	Permanent LAD ligation	CDCs	Rat	Syngeneic	25*10^5	Injected 1x in infarct area, 2x in border zone	N	PBS	Immediately after MI	4w	4w
Lan, 2012 [32]	NOD-SCID mice	F	adult	Permanent LAD ligation	Sca-1+ CSCs	Human	Xenogeneic	1*10^6	Injected in border zone	Y	PBS	Immediately after MI	2w, 8w	2w, 8w
Latham, 2013 [33]	NOD-SCID mice	M		Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected into borderzone and cardiac apex	Y	PBS	7 days after MI	21d, 28d	2w, 3w
Lee, 2011 [34]	Yucatan minipigs	F		2.5h LAD balloon occlusion	Cs, CDCs	Pig	Autologous	20x10^5	Injected in border zone	N	PBS	4w after MI	8w	4w
Li, 2009 [35]	FVB mice	F	8-10w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngenic	5*10^5	Injected in border zone	N	PBS	30min after MI	56d	56d
Li, 2010 [36]	SCID-Beige mice	M	10-12w	Permament LAD ligation	Cs/CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	1w, 3w	1w, 3w
Li, 2011a [37]	C57BL6/J mice	F	11-12w	60-min coronary balloon occlusion	c-kit+ CSCs	Mouse	Syngeneic	4*10^5	Intracoronary infusion / Injected in border zone	N	PBS	48h after MI	37d	35d
Li, 2011b [38]	SCID mice	M	10-12w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Li, 2012 [39]	SCID-Beige mice	M	10-12w	Permanent LAD ligation	CDCs	Rat	Xenogeneic	4*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Liu, 2012 [40]	SCID-Beige mice	F	adult'	Permanent LAD ligation	Sca-1+ CSCs	Human	Xenogeneic	1*10^6	Injected in border zone	Y	PBS	Immediately after MI	1w, 2w, 3w, 4w	1w, 2w, 3w, 4w
Malliaras, 2012 [41]	BN rats, WKY rats	F	8-10w	Permanent LAD ligation	CDCs	Rat, Human	Allogeneic, Syngeneic, Xenogeneic	2*10^6	Injected in border zone	N	PBS	Immediately after MI	3w, 3m, 6m	3w, 3m, 6m
Malliaras, 2013a [42]	Yucatan minipigs		adult	2.5h LAD balloon occlusion	CDCs	Pig	Allogeneic	12.5*10^6	Intracoronary infusion	N	Vehicle	2-3w after MI	10-11w	8w
Malliaras, 2013b [43]	MerCreMer/Zeg mice		6-8w	Permanent LAD ligation	CDCs	Mouse	Allogeneic	2*10^5	Injected in border zone	N	PBS	Immediately after MI	5w	5w
Malliaras, 2014 [44]	bitransgenic MerCreMer/ZEG and B6129SF1/J		8-12w	Permanent LAD ligation	CDCs	Mouse	Allogeneic	2*10^5	Injected in border zone	N	PBS	Immediately after MI	5w	5w
Martens, 2011 [45]	SCID-Beige mice		8-10w	Permanent LAD ligation	CDCs	Rhesus monkey	Xenogeneic	1*10^6	Intramyocardial injection	Y	PBS	Immediately after MI	1w, 4w	1w, 4w
Matsuda, 2014 [46]	F344/NJcl-rnu/rnu rats	F	8w	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	8*10^4	Injected in border zone	N	PBS	Immediately after MI	1w, 2w, 3w	1w, 2w, 3w
Mayfield, 2014 [47]	NOD-SCID mice	M	8-9w	Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	1*10^5	Injected into borderzone and cardiac apex	Y	PBS	1w after MI	1w, 2w, 3w, 4w	0w, 1w, 2w, 3w
Messina, 2004 [48]	SCID-Beige mice			Permanent LAD ligation	Cs	Human	Xenogeneic	4*10^5	Injected in border zone	Y	PBS	Immediately after MI	18d	18d
Mishra, 2011 [49]	Nude rats	M		Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^6	Injected in infarct and border zone	Y	PBS	Immediately after MI	1w, 4w	1w, 4w
Mohsin, 2012 [50]	SCID mice			Permanent LAD ligation	c-kit+ CSCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	Vehicle	Immediately after MI	multiple, last 20w	multiple, last 20w
Ong, 2014 [51]	NOD-SCID mice	F	8-10w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Allogeneic	1*10^6	Injected in border zone	Y	Saline	immediately after MI	6w	6w
Oskouei, 2012 [52]	NOD-SCID mice	F	8-10 weeks	Permanent LAD ligation	c-kit+ CSCs	human	Xenogeneic	36*10^3	Intramyocardial injection	Y	PBS	Immediately after MI	48h and 1w, 2w, 4w, 8w	48h and 1w, 2w, 4w, 8w
Padin-Iruegas, 2009 [53]	Fischer 344 rats	F	3m	Permanent LAD ligation	c-kit+ CSCs	Rat	Syngeneic	1*10^5	Injected in border zone	N	PBS	Immediately after MI	4w	4w
Quijada, 2015 [54]	FVB mice	F	11w	Permanent LAD ligation	c-kit+ CSCs	mouse	Syngeneic	1*10^5	Injected in border zone	N	PBS	Immediately after MI	multiple, last 18w	multiple, last 18w
Rota, 2008 [55]	Fischer 344 rats	F	3m	Permanent LAD ligation	c-kit+ CSCs	Rat	Syngeneic	4*10^4	Injected in border zone	N	Saline	20d after MI	34d	16d
Ryzhov, 2013 [56]	C57BL/6 mice	M	10-12w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngeneic	2.5*10^5	Injected in border zone	N	PBS	Immediately after MI	1w, 2w, 3w, 4w	1w, 2w, 3w, 4w
Sharma, 2015 [57]	Rats	male		Permanent LAD ligation	CDCs	Human	xenogeneic	1*10^6	Injected in border zone	Y	Medium	Immediately after MI	1w, 4w	1w, 4w
Shen, 2012 [58]	SCID-Beige mice	M	adult	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^4, 5*10^4, 1*10^5, 5*10^5	Injected in border zone	Y	PBS	Immediately after MI	3w	3w
Simpson, 2012 [59]	Nude rats	M		Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^6	Injected in infarct and border zone	Y	Fibroblasts	10min after ligation	1w, 4w	1w, 4w

Smith, 2007 [60]	SCID-Beige mice	M	10-20w	Permanent LAD ligation	CDCs	Human & Pig	Xenogeneic	1*10^5	Injected in border zone	Y	PBS	Immediately after MI	20d	20d
Smits, 2009 [61]	NOD-SCID mice	M	10-12w	Permanent LAD ligation	Sca-1+ CSCs	Human	Xenogeneic	5*10^5	Injected in border zone	Y	PBS	Immediately after MI	4w, 12w	4w, 12w
Tang, 2010 [62]	Fischer 344 rats	F	3m	2h LAD occlusion	c-kit+ CSCs	Rat	Syngeneic	1*10^6	Aortic root infusion	N	Saline	30d after MI surgery	65d	35d
Tang, 2015 [63]	Fischer 344 rats	F	10-12w	90-min LAD occlusion	c-kit+ CSCs	Rat	Syngeneic	0.3*10^6, 0.75*10^6, 1.5*10^6, 3*10^6	Intracoronary injection	N	PBS	4h after MI	5w	5w
Tateishi, 2007a [64]	C57BL/6 mice		12-24w	Permanent LAD ligation	CDCs	Mouse	Allogeneic	5*10^5	Injected in border zone	N	PBS	1h after MI	4w	4w
Tateishi, 2007b [65]	NOD-SCID mice		12-24w	Permanent LAD ligation	CDCs	human	Xenogeneic	3*10^5	Injected in border zone	Y	PBS	1hr after MI	2w, 4w	2w, 4w
Tseliou, 2013 [66]	WKY rats, BN rats	F	6-7w	Permanent LAD ligation	Cs	Rat	Allogeneic, Syngeneic, Xenogeneic	2*10^6	Injected in border zone	N	PBS	Shortly after MI	1w, 3w, 3m, 6m	1w, 3w, 3m, 6m
Tseliou, 2014a [67]	WKY rats	F	6-8w	Permanent LAD ligation	CDCs	Rat	Syngeneic	2*10^6	Injected in border zone	N	PBS	Immediately after MI	7d, 21d	7d, 21d
Tseliou, 2014b [68]	WKY rats	F	6-8w	Permanent LAD ligation	Cs	Rat	Syngeneic	2*10^6	Injected in border zone	N	PBS	1m after MI	4w, 5w, 8w, 28w	1w, 1m, 6m
Vandergriff, 2014 [69]	WKY rats	F		Permanent LAD ligation	CDCs	Rat	Syngeneic	5*10^5	Intracoronary injection	N	PBS	Immediately after MI	3w	3w
Wang, 2006 [70]	Balb/c mice	F	10-12w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngeneic	1*10^6	Injected in border zone	N	PBS	Immediately after MI	1w, 2w, 3w	1w, 2w, 3w
Wang, 2014 [71]	Balb/c mice	F	10-12w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngeneic	1*10^6	Injected into borderzone	N	PBS	Immediately after MI	4w	4w
Welt, 2013 [72]	Mongrel dogs	M	7-18m	LAD and diagonal branches ligation	c-kit+ CSCs	Dog	Autologous	16*10^5	Injected in border zone	N	PBS	6w after MI	6w, 30w	24w
Williams, 2013 [73]	Yorkshire swine			90-min LAD occlusion	c-kit+ CSCs	Human	Xenogeneic	1*10^6	Injected in border zone	Y	PBS	2w after MI	4w, 6w	2w, 4w
Winter, 2009 [74]	NOD-SCID mice	M	11-12w	Permanent LAD ligation	Sca-1+ CSCs	Human	Xenogeneic	4*10^5	Injected in infarct and border zone	Y	Medium	Immediately after MI	6w	6w
Xie, 2014 [75]	SCID-Beige mice	M	8-12w	Permanent LAD ligation	CDCs	Human	Xenogeneic	1*10^5	Injected in border zone	Y	Medium	Immediately after MI	3w	3w
Yan, 2012 [76]	C57BL/6 mice	?	10w	Permanent LAD ligation	c-kit+ CSCs	Mouse	Syngeneic	5*10^5	Intramyocardial injection	N	Medium	Immediately after MI	7d	7d
Ye, 2012 [77]	C57BL/6 mice	M	9m	Permanent LAD ligation	Cs, Sca-1+ CSCs	Mouse	Syngeneic	1*10^5, 1*10^6	Injected in infarcted myocardium	N	PBS	2d after MI	28d	25d
Yee, 2014 [78]	Yukatan minipigs	2M, 22F	9m	2.5h LAD balloon occlusion	Cs	Pig	Allogeneic	15*10^6, 45*10^6, 150*10^6	Transendocardial injection	N	Vehicle	4w after MI	8w	4w
Pivotal study	Yukatan minipigs	14M, 15F	10m	3h LAD balloon occlusion	Cs	Pig	Allogeneic	150*10^6	Transendocardial injection	N	Vehicle	8w after MI	16w	8w
Zakharova, 2014 [79]	SD rats	M	2m	Permanent LAD ligation	c-kit+ CSCs	Rat	Syngeneic	1*10^6	Right atrium infusion	N	Medium	21d after MI	42d	21d
Zhao, 2014 [80]	C57BL/6 mice	M	10-12w	Permanent LAD ligation	Sca-1+ CSCs	Mouse	Syngeneic	2*10^5	Injected in infarct and borderzone	N	Medium	30min after MI	2w	2w

Supplementary table I. Characteristics and study design of all included pre-clinical studies. Abbreviations: SCID - severe combined immunodeficiency ; WKY - Wistar Kyoto ; SD - Sprague-Dawley ; NOD - Non-obese diabetic ; BN - Brown Norway ; M - male ; F - female ; w - weeks; m - months
LAD - left anterior descending coronary artery

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Cell type	No. of studies (comparisons)	No. controls	No. treated	No. syngeneic/ allogeneic/xenogeneic	% immune-suppressed	Follow-up time mean*/median / (min-max) (days)	Time of administration mean*/median / (min-max) (hours)	Cell number mean*/median	Mouse / Rat
CDCs	32(44)	261	473	9/9/26	59% (26/44)	78/21 (14-168)	11/0 (0-168)	716.053 / 250.000	26/18
Cs	6(9)	62	112	3/1/5	33% (3/9)	77/25 (18-168)	86/0 (0-672)	1150830/ 1.000.000	4/5
c-kit$^+$ CSC	22(29)	271	326	16/0/13	41% (12/29)	33/35 (7-140)	106/5 (0-720)	369.138 / 300.000	16/13
Sca-1$^+$ CSC	13(13)	129	166	7/1/5	46% (6/13)	35/28 (14-84)	9/0 (0-48)	676.156 / 500.000	13/0

Supplementary Table II. Descriptive statistics of all small animal studies per cell type.
*Weighed mean based on to the share of each study in the meta-analysis

		IS/LV (%)		IS/AAR (%)		ESV (SMD)		EDV (SMD)		WT (SMD)		FS (%)	
		Effect size	p-value	Effect size	p-value	Effect size	p-value	Effect size	p-value	Effect size	p-value	Effect size	p-value
Cell type	CDC	-9.5 (-5.1 – -13.9)	0.17	-12.7 (-9.6 – -15.8)	**0.009***	-2.0 (1.4 – -5.4)	0.43	-0.9 (-0.5 – -1.4)	0.86	2.0 (1.4 – 2.5)	0.14	7.5 (3.6 – 11.3)	0.51
	Cs	-14.7 (-7.4 – -22.0)										5.2 (1.2 – 9.3)	
	C-kit	-5.2 (-1.8 – -12.2)		-3.4 (2.5 – -9.4)		-4.2 (-1.9 – -6.6)		-0.9 (-0.4 – -1.3)		1.3 (0.7 – 2.0)		6.9 (4.2 – 9.6)	
	Sca-1					-1.0 (5.8 – -7.8)		-0.6 (1.4 – -0.1)				3.9 (0.0 – 8.1)	
Immuno-suppression	Yes	-15.1 (-10.8 – -19.3)	**0.002***	-10.1 (-6.1 – -14.0)	0.44	-1.5 (2.0 – -5.1)	0.24	-0.9 (-0.4 – -1.3)	0.49	2.0 (1.4 – 2.6)	0.18	4.4 (1.6 – 7.2)	0.15
	No	-5.8 (-2.2 – -9.3)		-12.9 (6.5 – -19.3)		-4.0 (-1.8 – -6.2)		-0.8 (-0.4 – 1.2)		1.5 (0.9 – 2.0)		6.9 (4.9 – 8.9)	
Donor	Syngeneic	-6.0 (-0.9 – -11.2)	0.051			-4.4 (-2.0 – -6.7)	0.38	-0.8 (-0.3 – 1.3)	0.97	1.6 (0.9 – 2.2)	0.24	7.6 (5.5 – 9.7)	0.028
	Allogeneic					-2.4 (2.9 – -7.7)		-0.9 (0 – -1.7)				7.6 (3.0 – 12.2)	
	Xenogeneic	-12.9 (-8.3 – -17.5)				-1.5 (2.0 – -5.0)		-0.8 (-0.4 – -1.3)		2.1 (1.5 – 2.8)		3.1 (0.5 – 5.7)	
Publication bias	Control									1.7 (0.9 – 2.5)	0.90		
	Ultimate									1.8 (1.2 – 2.3)			
Culture 2D/3D	2D												
	3D												
Cell morbidity	Healthy												
	Diseased												
Randomization	Yes												
	No												
Allocation concealment	Yes												
	No												
Blinding at assessment	Yes												
	No												
Ischemia model	Permanent												
	I/R												

Supplementary Table III. Meta-regression analyses for all secondary outcomes; infarct size in left ventricle (IS/LV), infarct size in area at risk (IS/AAR), end systolic volume (ESV), end diastolic volume (EDV), wall thickness (WT) and fractional shortening (FS). Values are in raw mean differences in percentages (%) or standardized mean differences (SMD). The number of variables used for analyses was the number of comparisons divided by 10. A subgroup needed at least 5 different measurements to be included; in analyses with >2 groups, a group with <5 comparisons was excluded from that specific analysis. Taking multiple testing into account, we applied a Bonferroni-Holmes correction for number of variables, therefore considering a p-value of 0.017 significant.

(A)

Author	Section describing drop-outs or mortality?	Number of drop-outs and reason specified? (all animals)	Number of used animals in M&M (of primary outcome groups)?	Number of animals in results (primary outcome)?	Number of animals match?	Risk assessment
Avolio, 2014	No	N/A	52	52	Yes	Low
Avolio, 2015	No	N/A	40	6-7 per group	Unknown	Unknown
Barth, 2012	No	N/A	No	23	Unknown	Unknown
Beltrami, 2003	No	N/A	13/14	No	Unknown	Unknown
Bolli, 2013	Yes	8, yes	21	21	Yes	Low
Bonios, 2011	No	N/A	14	No	Unknown	Unknown
Cai, 2015	No	N/A	No	27	Unknown	Unknown
Campan, 2011	No	N/A	13	13	Yes	Low
Carr, 2011	No	N/A	14	No	Unknown	Unknown
Chan, 2015	No	N/A	No	14	Unknown	Unknown
Cheng, 2010	No	N/A	16	7-8 per group	No	High
Cheng, 2012a	No	N/A	82	9 per group	Unknown	Unknown
Cheng, 2012b	No	N/A	61	61	Yes	Low
Cheng, 2014a	No	N/A	44	44	Yes	Low
Cheng, 2014b	No	N/A	75	19-24 per group	Unknown	Unknown
Chimenti, 2010	No	N/A	At least 3 per group	Unclear	Unknown	Unknown
Cho, 2012	No	N/A	No	24	Unknown	Unknown
Crisostomo, 2015	Yes	2, yes	17	17	Yes	Low
D'amario, 2011	No	N/A	No	15	Unknown	Unknown
Davis, 2010	No	N/A	14	6-8 per group	Yes	Low
Dawn, 2005	Yes	27, yes	41	41	Yes	Low
De Couto, 2015	No	N/A	No	15	Unknown	Unknown
den Haan, 2012	Yes	28, no	23	23	Yes	Low
Fischer, 2011	Yes	No	34	15	Unknown	Unknown
Gallet, 2015	Yes	5, yes	14	14	Yes	Low
Goichberg, 2011	No	N/A	No	17	Unknown	Unknown
Hong, 2014	No	N/A	No	16	Unknown	Unknown
Jackson, 2015	No	N/A	18	8-9 per group	Unknown	Unknown
Johnston, 2009	Yes	3, yes	16	13	Yes	Low
Kim, 2013	No	N/A	4-9 per group	13	Yes	Low
Kim, 2015	No	N/A	No	14	Unknown	Unknown
Lan, 2012	No	N/A	30	30	Yes	Low
Latham, 2013	No	N/A	10-15 per group	22	No	High
Lee, 2011	yes	3, yes	29	23	No	High
Li, 2009	No	N/A	45	45	Yes	Low
Li, 2010	No	N/A	48	48	Yes	Low
Li, 2011a	Yes	42, yes	No	49	Yes	Low
Li, 2011b	No	N/A	20	20	Yes	Low
Li, 2012	No	N/A	28	No	Unknown	Unknown
Liu, 2012	No	N/A	50	47	No	High
Malliaras, 2012	Yes	31, no	Unclear	160	Unknown	Unknown
Malliaras, 2013a	No	N/A	No	18	Unknown	Unknown
Malliaras, 2013b	No	N/A	No	10	Unknown	Unknown
Malliaras, 2014	Yes	4, yes	10	No	Yes	Low
Martens, 2011	Yes	9, no	28	25	Yes	Low
Matsuda, 2014	No	N/A	No	9-11 per group	Unknown	Unknown
Mayfield, 2014	No	N/A	No	11	Unknown	Unknown
Messina, 2004	Yes	No	No	10	Unknown	Unknown
Mishra, 2011	No	N/A	No	11	Unknown	Unknown
Mohsin, 2012	No	No	40	28	No	High
Ong, 2014	No	N/A	20	20	Yes	Low
Oskouei, 2012	Yes	9, no	46	10	Unknown	Unknown
Padin-Iruegas, 2009	No	N/A	No	20	Unknown	Unknown
Quijada, 2015	No	N/A	No	11	Unknown	Unknown
Rota, 2008	No	N/A	No	38	Unknown	Unknown
Ryzhov, 2013	No	N/A	No	6-7 per group	Unknown	Unknown
Sharma, 2015	No	N/A	No	12	Unknown	Unknown
Shen, 2012	No	N/A	No	5-6 per group	Unknown	Unknown
Simpson, 2012	No	N/A	No	13	Unknown	Unknown
Smith, 2007	No	N/A	No	22	Unknown	Unknown
Smits, 2009	Yes	3, no	20	17	Yes	Low
Tang, 2010	Yes	32, yes	47	47	Yes	Low
Tang, 2015	Yes	15, yes	No	31	Yes	Low
Tateishi, 2007a	No	N/A	No	16	Unknown	Unknown
Tateishi, 2007b	No	N/A	No	16	Unknown	Unknown
Tseliou, 2013	No	N/A	71	14	Unknown	Unknown
Tseliou, 2014a	No	N/A	110	No	Unknown	Unknown
Tseliou, 2014b	No	N/A	12	12	Yes	Low
Vandergriff, 2014	No	N/A	58	14	Unknown	Unknown
Wang, 2006	no	N/A	No	16	Unknown	Unknown
Wang, 2014	No	N/A	No	18	Unknown	Unknown
Welt, 2013	Yes	9, yes	19	19	Yes	Low
Williams, 2013	No	N/A	10	10	Yes	Low
Winter, 2009	No	N/A	30	No	Unknown	Unknown
Xie, 2014	No	N/A	No	6-7 per group	Unknown	Unknown
Yan, 2012	No	N/A	10	No	Unknown	Unknown
Ye, 2012	No	N/A	27	24	Unknown	Unknown
Yee, 2014	Yes	26, yes	63	63	Yes	Low
Zakharova, 2014	Yes	No	18	18	Yes	Low
Zhao, 2014	No	N/A	12	12	Yes	Low

(B)

	Large animals	Small animals	Total
Low	8	21	29
Unknown	0	46	46
High	1	4	5
Total	**9**	**71**	**80**

Supplementary table IV. Attrition bias was assessed using the checklist provided by SYRCLE. (A) Risk of attrition bias was assessed based on the information given about the mortality and reason of drop-outs. In this case, all animals were included in the assessment. If this information was missing, the assessment was based on matching amounts of animals mentioned in the materials & methods (M&M) and in the results of the primary outcome. (B) Summary of the tabel in A, with a distinction between large and small animals.

8

Combined meta-analysis of preclinical cell therapy studies shows overlapping effect modifiers for multiple diseases

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In preparation

ABSTRACT

Introduction

Cell therapy has been studied for many years in many different research domains. For hematological cancers, bone marrow transplants are well established clinical therapies. Cellular replacement of damaged solid tissues is at a much earlier stage of development, with much still to be understood. Systematic review and meta-analysis are widely used to aggregate data and find important patterns of results within research domains. In this paper, we use these tools across research areas to look for common biological denominators affecting therapeutic effect size in preclinical stem and progenitor cell therapy studies for renal, neurological and cardiac disease.

Methods

We used datasets of five previously published meta-analyses investigating the potential of stem and progenitor cell therapy in preclinical models of chronic kidney disease, spinal cord injury, stroke, and cardiac ischemic disease. We transformed all primary outcome measures to ratios of means to permit direct comparison across disease areas. Pre-specified variables of interest were species, use of immunosuppression and the characteristics of the cellular interventions themselves.

Results

The five datasets from 506 publications yielded data from 13,638 animals. There was a strong inverse relationship between effect size and size of the experimental animal. Cell type seemed to influence efficacy, with no clear trend for certain cell types being superior across all disease models. Efficacy was not affected by source of the cell therapeutics. Immunosuppression showed a negative influence in spinal cord injury and possible positive effects when introduced genetically in cardiac ischemic models.

Conclusions

All preclinical cell therapy studies seem to be affected by the same decrease in effect size when using larger animals compared to rodents. This has important implications for the translation of cellular therapeutics to the clinic. This translational failure might be attributed to the biological activity of the cell product, but might also be caused by other sources like study setup and biases.

ABBREVIATIONS

CKD	chronic kidney disease
SCI	spinal cord injury
MI	myocardial infarction
CSC	cardiac stem cell

Introduction

Stem and progenitor cells have emerged in many different areas of research and medicine. They have the capacity to replace damaged tissue, to be used for study of human development, and disease, as well as a test bed for discovering new drugs and gene therapies. Cellular products as a therapeutic have raised new paradigms of regeneration for many organs, especially organs that do not heal easily, like the brain, heart, kidney, cartilage and eye.[1] In light of the overwhelming positive results seen in preclinical studies, multiple research fields are translating cell therapy into the clinic. The rationale for transplanting these stem and progenitor cells is multimodal, potentially replacing lost tissue and predominantly supporting the surviving cells through paracrine mechanisms or modulation of the immune response.[2] These mechanisms are being continuously explored, pointing towards soluble growth factors, cytokines and extracellular vesicles as major mediators in these processes.

Preclinical studies are always the starting point for such promising new therapies. Animal experiments allow exact control of experimental conditions and access to post-mortem material with fewer restrictions than human trials, while maintaining the complexity of a whole organism. Defined preclinical models of disease have been standardized to a large extent and are ideally comparable across research centers. Rodent models are most frequently used, as rodents are easy to handle, cost-effective to maintain, have a short generation span and the availability of inbred strains allows great experimental reproducibility and stable breeding of genetically modified animals. On the other hand, larger animal models show greater similarity to human physiology and are often used as an intermediate 'verification step' in the translational axis towards human application.[3]

In the preclinical application of cell therapies, multiple meta-analyses have been performed on studies using renal[4], neurological[5,6], and cardiac[7,8] disease models. Since the mode of action hypothetically is similar for these stem and progenitor cells, common effects for these and future cell therapy research fields could be present in these datasets. Furthermore, an overview of the different stages of cell therapy initiatives in disease models will give us an overview of the stages in which research fields currently are and will perhaps allow extrapolation to the future. In this paper, we focus on potential common denominators in these studies to ultimately find certain overlapping cell therapy characteristics across disease entities. Next, we searched for all phase III trials in these diseases, to see which research fields are getting closest to clinical application.

Methods

We used the original data of five previously published preclinical meta-analyses on the effect of stem and progenitor cell therapy in chronic kidney disease (CKD)[4], stroke[5], spinal cord injury (SCI)[6], and myocardial infarction (MI).[7,8] For the MI datasets, these included both acute and chronic models of ischemic cardiomyopathy. To our knowledge, these datasets are the only systematic reviews of preclinical cell therapy applications. We converted all outcome measures to ratios of means[9] (ROMs) to provide a standardized measure of effect size across the outcomes in different models: (among others) blood pressure and urinary protein[4], neural infarct volume and motor or sensory outcome[5,6] and ejection fraction measurements for cardiac function[7,8], respectively.

Variables of interest were species/animal size, cell origin (autologous/syngeneic vs allogeneic vs xenogeneic), cell type (bone marrow-derived vs mesenchymal stem cells vs blood-derived vs tissue-resident cell vs pluripotent cell (e.g. iPSCs or ESCs) vs other cell types) and the use of immunosuppression (drug-induced or genetic). For cell type we combined various cell types in one 'other cell type' variable, including amniotic fluid cells, dermal cells and hair follicle cells. Variables were retrieved from the original publications or recoded if not already present in the desired format.

The search for Phase III trials was performed on the website www.clinicaltrials.gov. Search terms were "phase 3 stem cells" in combination with the disease of interest ("Renal Failure", "Kidney Failure", "Amyotrophic Lateral Sclerosis", "Motor Neuron Disease", "Parkinson", "Alzheimer", "Huntington", "Stroke", "Spinal Cord Injury", "Myocardial Infarction" and "Heart Failure"). All retrieved studies were screened for their relevance. Trial registration numbers of relevant studies were recorded, regardless of their status.

Statistical analysis

Random-effects meta-analysis was performed for all datasets. Univariable meta-regression was performed for the chosen variables with the log of our generated ROMs to assess the influence on overall effect size. Log(ROMs) were transformed back in figures for proper visualization. Multivariable analysis with all variables of interest was performed for every dataset individually and for all datasets combined. In this final analysis for all datasets, we also corrected for the datasets by putting this in as an additional variable. Post-hoc testing was performed using a Wald-test. Three studies from one MI dataset[8] were removed for this combined analysis, as these were also present in another dataset.[7] For the multivariable analyses, p-values for the individual variables within the multivariable meta-regression are reported. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using R version 3.1.2[10] with the additional metafor package.[11]

Results

The combined data from 5 datasets yielded 506 publications with a total of 13,638 included animals. Descriptive statistics of all studies are depicted in Table 1.

The animals used in these studies range from: mice and rats, most commonly used in models of CKD, stroke, SCI and MI; to rabbits, gerbil marmosets in the neurological studies; to pigs and sheep predominantly used in models of MI. Regardless of the primary outcome measure used, stem and progenitor cell therapy appeared to improve the outcome in all disease models. (Table 1).

	Papazova et al.[4]	Lees et al.[5]	Antonic et al.[6]	Jansen of Lorkeers et al.[7]	Zwetsloot et al.[8]
Disease type	Nephrology	Stroke	SCI	MI (large animals, all cell types)	MI (all animals, cardiac stem cells)
No. of studies	71	117	156	82	80
No. of comparisons	-	192	319	125	109
No. of animals	1813	2704	5736	1415	1970
Primary outcome(s)	Blood pressure & urinary protein	Infarct volume & Neurobehavioral	Neurobehavioral outcomes	Cardiac function (EF)	Cardiac function (EF)
Overall effect size	0.60(0.34-0.87)*	24.8%(21.5-28.1)	27.3% (25.1-29.4%)	8.3%(7.1–9.5%)	10.7%(9.4-12.1)

Table 1. Summary of baseline characteristics of included systematic reviews.*primary outcome for blood pressure converted to SMD

Species

We stratified our dataset according to animal size/species. Small animals such as mice and rats appear to consistently have larger effect sizes across all disease models, compared to larger animals such as dog, pigs or marmosets (Figure 1). Only the SCI dataset did not show the same trend (Figure 1D).

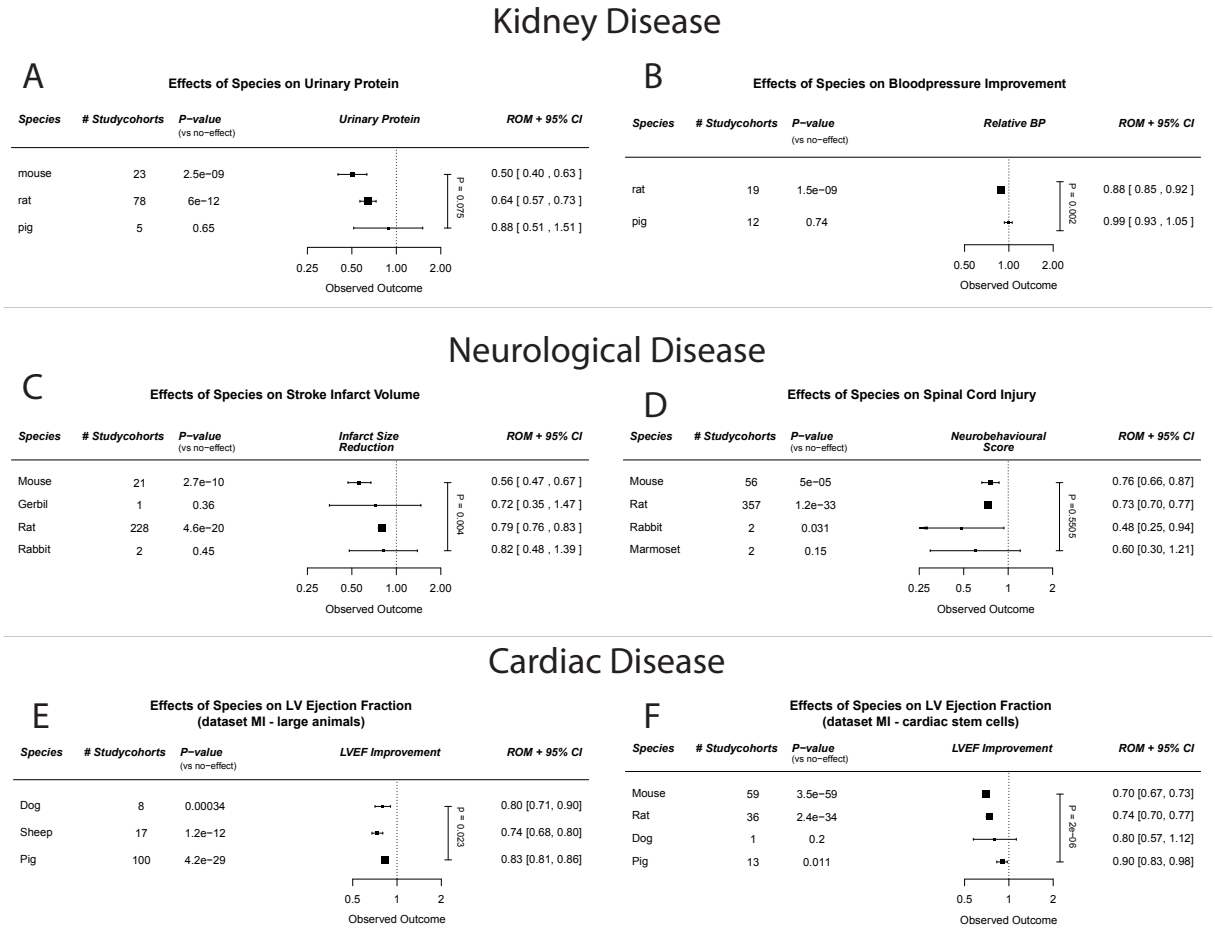

Figure 1. Meta-regression analyses with regards to cell therapy efficacy in different species quantified by (A) urinary protein (CKD), (B) blood pressure difference (CKD), (C) infarct size (stroke), (D) neurobehavioral scores (SCI), (E) ejection fraction (large animal MI studies), (F) ejection fraction (CSC MI studies). Outcomes are expressed in ROMs. Vertical p-value represents the total meta-regression. Horizontal p-values are significance compared to an assumed 'no effect'.

Cell type

Cell type did not seem to influence any outcome in CKD (Figure 2A-B). In the stroke data, cell type did explain part of the heterogeneity, with brain-specific cell types performing worse, especially compared to pluripotent cells and MSCs (Figure 2C). In the SCI dataset, brain-specific cell types again performed worst, with blood-derived cells and a collection of different 'other' cell types performing slightly better (Figure 2D). In large animal MI studies, there was no difference in efficacy between different cell types (Figure 2E). In the small animal MI studies using cardiac stem cells (CSCs), cardiosphere-derived cells were deemed most superior compared to Sca-1^{+} cells (Figure 2F).

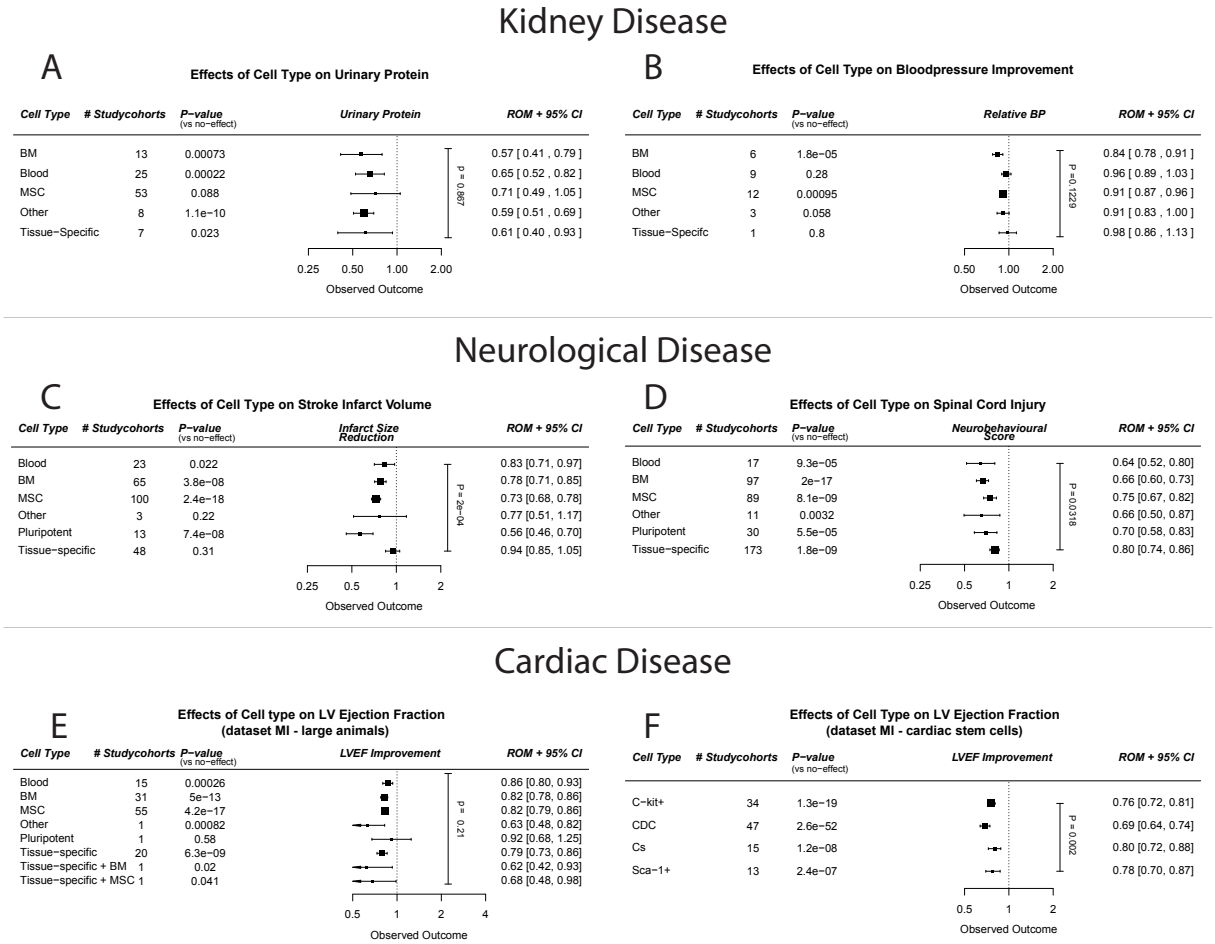

Figure 2. Meta-regression analyses with regards to cell therapy with different cell types quantified by (A) urinary protein (CKD), (B) blood pressure difference (CKD), (C) infarct size (stroke), (D) neurobehavioral scores (SCI), (E) ejection fraction (large animal MI studies), (F) ejection fraction (CSC MI studies). Outcomes are expressed in ROMs. Vertical p-value represents the total meta-regression. Horizontal p-values are significance compared to an assumed 'no effect'.

Cell source

Cell source did not appear to affect the outcomes in the CKD, SCI and large animal MI datasets (Figure 3A-B,D-E). In the stroke data, the small number of studies using autologous cells seemed more efficacious compared to allogeneic and xenogeneic studies (Figure 3C). In the cardiac dataset with CSCs, autologous cells seemed less efficacious compared to the other cell types (Figure 3F).

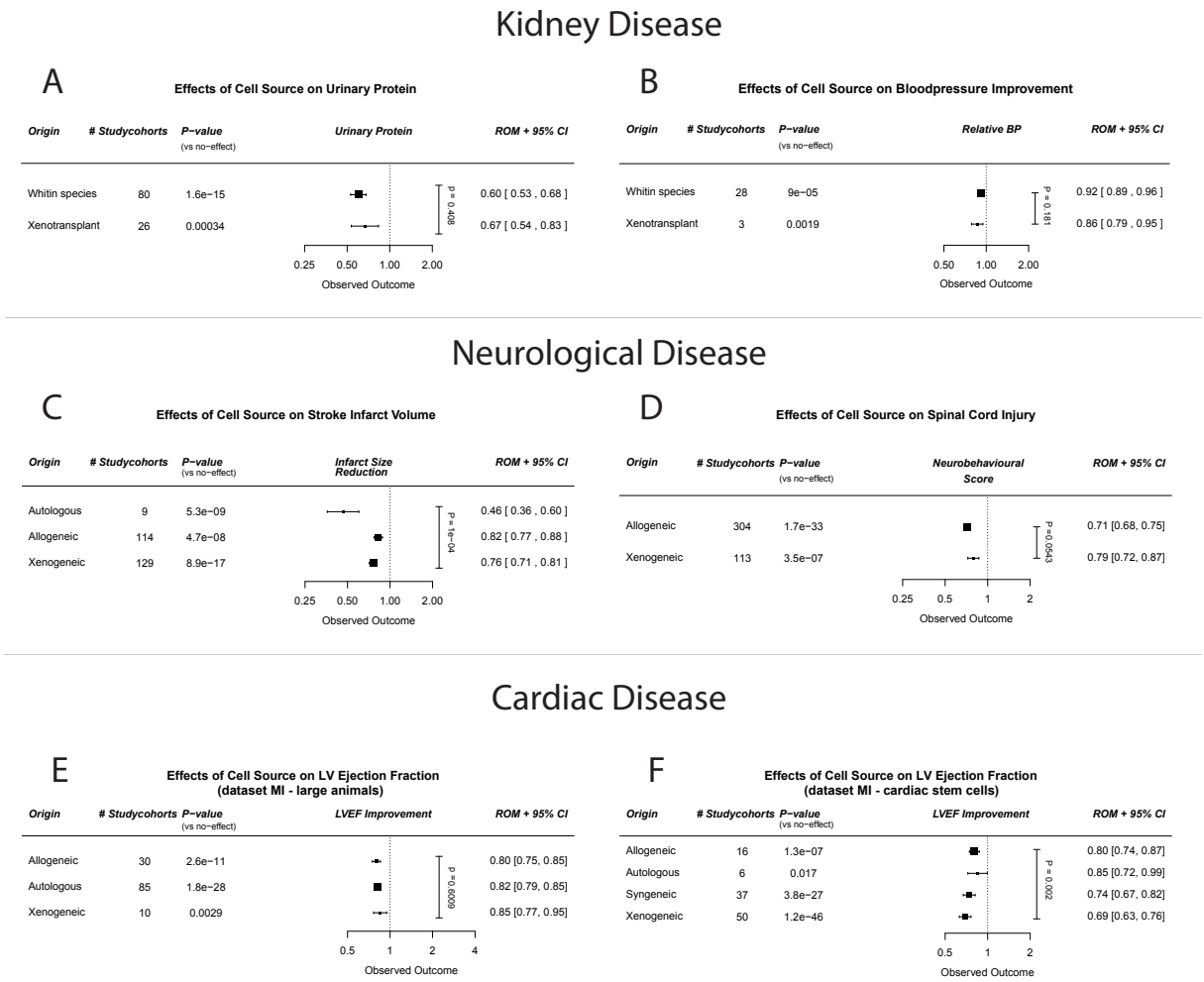

Figure 3. Meta-regression analyses with regards to cell therapy from different cell sources quantified by (A) urinary protein (CKD), (B) blood pressure difference (CKD), (C) infarct size (stroke), (D) neurobehavioral scores (SCI), (E) ejection fraction (large animal MI studies), (F) ejection fraction (CSC MI studies). Outcomes are expressed in ROMs. Vertical p-value represents the total meta-regression. Horizontal p-values are significance compared to an assumed 'no effect'.

Immunosuppression

The use of Cyclosporin A seems to have a negative influence on neurobehavioral scores after SCI (Figure 2C). In small animal models from the CSC dataset, it seems that genetically-modified immunodeficient mice do better, compared to immunocompetent animals (Figure 2E). In the other datasets, immunosuppression did not show any beneficial or detrimental effects. For the CKD dataset and blood pressure outcomes, there were no studies using any type of immunosuppression.

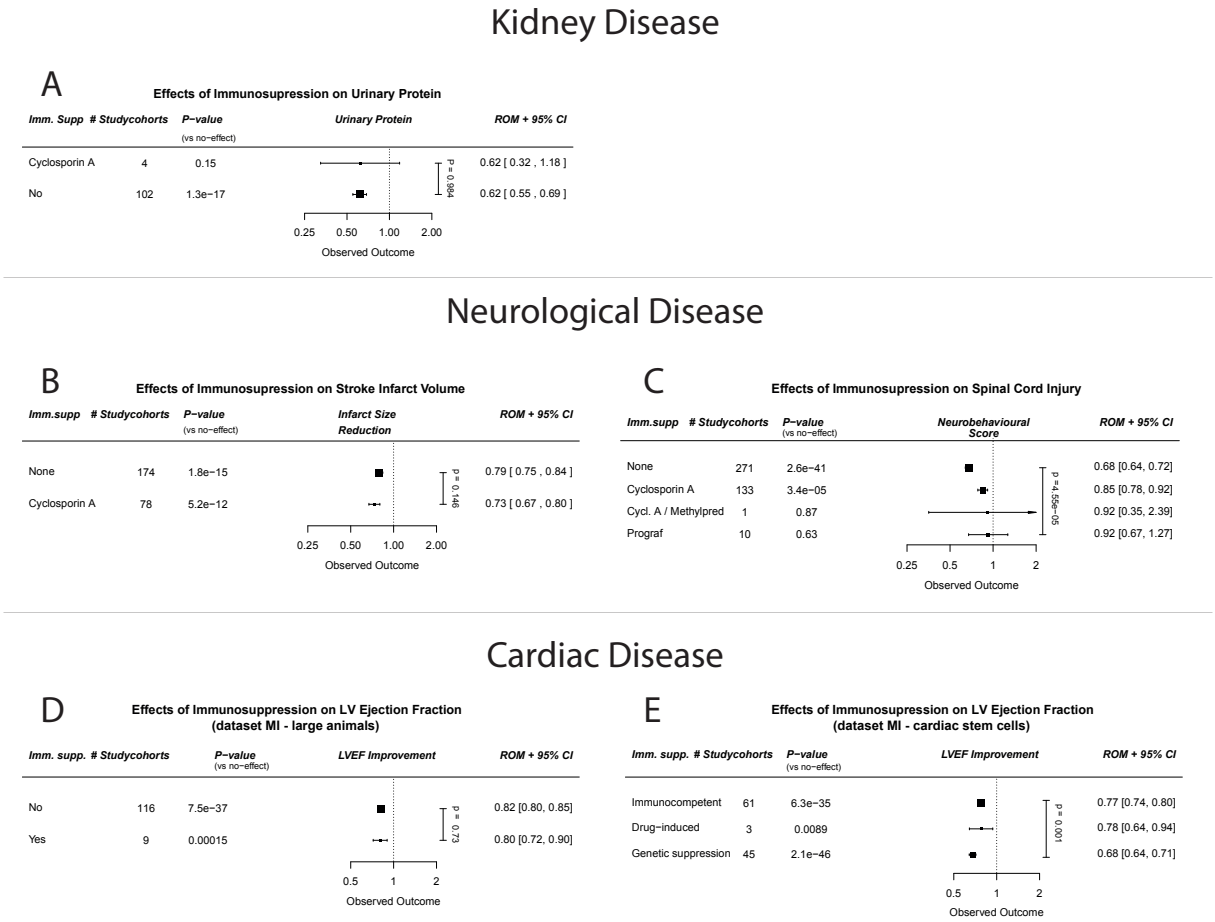

Figure 4. Meta-regression analyses with regards to cell therapy stratified for the use of immunosuppression, quantified by (A) urinary protein (CKD), (B) infarct size (stroke), (C) neurobehavioral scores (SCI), (D) ejection fraction (large animal MI studies), (E) ejection fraction (CSC MI studies). Outcomes are expressed in ROMs. Vertical p-value represents the total meta-regression. Horizontal p-values are significance compared to an assumed 'no effect'.

Multivariable analyses of individual and combined datasets

Next, we analysed all datasets multivariably, combining all variables of interest (Table 2). Most effects remained present when individual datasets were multivariably assessed, although the effect of cell origin and immunosuppression disappeared in the MI-CSC dataset. The multivariable analysis of all datasets combined, revealed that animal size (p=0.03) and cell type (p=0.02) seem to have comparable influences and common directions of effects in all datasets. For species, this was mainly a significant difference between pig and mouse models, favouring murine studies in terms of efficacy (p=0.01 for post-hoc testing). For cell type, this was apparent for tissue-specific cells, being less efficacious compared to pluripotent cells (p=0.006), bone marrow-derived cells (p=0.001) and mesenchymal stem cells (p=0.001).

	Papazova et al.				Lees et al.		Antonic et al.		Jansen of Lorkeers et al.		Zwetsloot et al.		ALL DATASETS*	
	Urinary protein		Blood pressure											
	univar	multivar	univar	multivar	univar	multivar	univar	multivar	univar	multivar	univar	multivar	univar	multivar
Animal size	0.08	0.10	**0.002**	**0.02**	**0.004**	**0.007**	0.55	0.44	**0.02**	0.10	**<0.0001**	**0.0027**	**0.0001**	**0.03**
Cell type	0.87	0.85	0.12	0.09	**0.0003**	**0.0001**	**0.03**	0.41	0.21	0.54	NA	NA	0.08	**0.02**
Cell origin	0.41	0.49	0.18	0.59	**0.0007**	**0.008**	0.05	0.29	0.60	0.31	**0.002**	0.81	0.88	0.38
Immunosupp	0.98	0.90	NA	NA	0.15	0.13	**0.0005**	**0.01**	0.73	0.24	**0.001**	0.32	**0.01**	0.15
(datasets)											*with extra correction for the different datasets		**<0.0001**	**0.006**

Table 2. p-values for all univariable and multivariable analyses of individual datasets and the combination of all datasets. NA= not applicable, either because of no immunosuppression being used (Papazova et al.) or because of the use of tissue-resident cells only (Zwetsloot et al.).

Current phase III trials in stem and progenitor cell therapy

Since we wanted to know the stage in which the different cell therapy fields are right now, we searched for all phase III trials in each disease. Our search retrieved 35 phase III trials, of which 29 were in the cardiac field (Table 3). The other 6 trials are in different neurological diseases. No phase III studies for kidney diseases were found through our search.

Disease type	# Phase III studies	Trial codes
Nephrology		
Renal Failure	0	
Kidney Failure	0	
Neurodegenerative Disorders		
Amyotrophic Lateral Sclerosis/ Motor Neuron Disease	1	NCT01933321
Parkinson's Disease	0	
Alzheimer's Disease	0	
Huntington's Disease	0	
Acute Neurological Disorders		
Stroke	2	NCT01716481, NCT02849613
Spinal Cord Injury	3	NCT02481440, NCT01676441, NCT01873547
Cardiac Disease		
Myocardial Infarction	15	NCT02672267, NCT01652209, NCT0116775, NCT01394432, NCT00725738, NCT01392105, NCT00279175, NCT01569178, NCT00350766, NCT00501917, NCT00950274, NCT01187654, NCT02323620, NCT00363324, NCT00684060
Heart Failure	14	NCT00526253, NCT01693042, NCT00462774, NCT01759212, NCT00747708, NCT02033278, NCT00841958, NCT00743639, NCT01753440, NCT00128258, NCT02032004, NCT00462774, NCT00333827, NCT00383630

Table 3. Summary of all current cell therapy clinical trials in phase III in the fields of nephrology, neurology and cardiology (www.clinicaltrials.gov search on 10-12-2016).

Discussion

In this paper, we show that common experimental choices show universal trends in preclinical stem and progenitor cell therapy studies in kidney, neurological and cardiac disease models through meta-analysis. These choices seem to affect common translation and might need attention to optimize our research and have accurate expectations of tested therapeutics. **Animal size** most strikingly affected the efficacy of cell therapy in all our analyses, with decreasing effectiveness as animal size increased. This decrease in effect size is most apparent going from rodents to larger mammals, but could in some datasets already be seen in the transition from mice to rats. This again confirms the need of rigorous large animal trials, since these animals more closely resemble human anatomy, physiology and ultimately reaction to therapy.[3] However, we are not sure if it's the actual animal size or the study quality difference that affects efficacy, as large animal studies also tend to resemble human clinical trials better with regards to blinding, randomization and susceptibility to bias.

Interestingly, for **cell type** in almost all cases tissue-specific cell types (e.g. a progenitor cell that resides in the organ of interest) did not show clear benefits over more easily obtained cells like bone marrow-derived cells, mesenchymal stem cells, circulating cells or pluripotent cells in these analyses. In our multivariable analysis of all datasets combined, tissue-specific cells even showed less efficacy compared to other cell products, although this might be predominantly driven by the neurological datasets. Interestingly, the modes of action might also be different for the different cell types under study, as a paracrine cell and a self-integrating 'residential' cell type could benefit a damaged organ differently. In the cardiac field, new preclinical studies are now hinting towards a superior effect of combinations of progenitor cell types, hypothetically making use of multiple cell-specific abilities.[12-15]

In all these different disease models, stem and progenitor cell therapy seems to give a comparable gain of function, regardless of **cell origin**. Like shown previously in the cardiac field, xenogeneic cells might show less benefit than allogeneic or autologous/syngeneic cell sources.[16] Allogeneic and autologous cells have shown comparable benefits, with the same proposed mechanisms[16], as is confirmed by our analyses.

Immunosuppression has shown to be of influence in the used datasets for stroke and SCI. In cardiac disease, cyclosporine has also been proposed as an agent that might affect both disease outcome and cell therapy.[17] However, In our combined datasets and multivariable analyses, we could not confirm a common effect of drug-induced immunosuppression, which was also not seen when directly studied on tissue-specific cardiac stem cells.[18] The effects we see do not show the same trends across different diseases.

Limitations

Of course, we should be careful with the interpretation of these results, as effect modification through known and unknown variables can always cause significance without true correlative relevance in these datasets. A possible solution for a portion of these effects could be multivariable meta-regression analyses, as shown in this paper. Some effects are reduced when correcting for other variables of interest, such as the effect of immunosuppression and cell origin in the MI-CSC dataset. Furthermore, one could also correct for quality and bias-introducing variables through

quality scores and the recorded use of randomization, blinding, etc. It is likely that there are still numerous other factors on top of quality indicators that influence the outcomes of these studies, making the interpretation of our results difficult as always.
Another factor to consider is our data conversion to ROMs, potentially introducing variability and distortions in some outcome measurements. While this could definitely be the case, our univariable ROM outcomes mostly reflected the known direction of effects from the original publications when investigating heterogeneity, suggesting the same effects and trends in these transformed datasets. Furthermore, through the use of ROMs we felt it was more appropriate to combine all these datasets, which would otherwise be less of an option due to different outcome measures.

Stem and progenitor cell therapy research in different stages of research

Table 3 summarises all current and completed phase III trials for stem and progenitor cell therapy in fields of nephrology, neuroscience (both neurodegenerative and acute disease), MI and heart failure. Although we found clear effects of stem and progenitor cell therapy and common factors affecting all these different diseases, some research areas are already reaching clinical end-products, while others are still in the process of passing preclinical phases. Reasons for this discrepancy remain to be elucidated, although factors like interest and funding might play a role. Current success stories in other disease entities not mentioned here, include cellular retinal transplants[19] and application of stem and progenitor cells or newly generated cartilage in degenerated joints.[20]

What can we learn from this?

In light of the questioned translatability of rodent models in cell therapy research[21], these current analyses again confirm the decrease in effect size across multiple disease models, when increasing animal size, as indicated for the heart.[8] Cell source and cell type only marginally influence efficacy, while immunosuppressive effects cannot be generalized to all diseases studied. This info calls for standardization of animal models and therapeutic approaches in all research areas, for which both the neurological and cardiac field have put forth first initiatives.[22-25] Trying to generalize meta-analyses of cell therapy preclinical studies might be of additive values to these, as these diseases vary widely and therefore can show both overlapping and different effects. In our current analyses, evident similarities imply effects of different species irrespective of disease model.

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9

The natural course of large animal myocardial infarction models: a systematic review and meta-analysis

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ABSTRACT

Aims

Large animal models are essential for the development of novel strategies for the treatment of myocardial infarction (MI). To improve translational success of interventions and further optimize reproducibility, extensive knowledge regarding the influence of experimental design on primary outcome measurements is mandatory. Meta-analysis of preclinical studies can increase methodological quality and identify determinants that affect outcome measurements. The aim of this study was to systematically investigate which factors independently influence these outcome measurements in large animal models.

Methods

We used all control animal-data from two independent meta-analyses of large animal MI models. We performed univariable and multivariable meta-regression to analyze whether relevant variables influenced infarct size as a ratio of the area at risk (IS/AAR), infarct size as a ratio of the left ventricle (IS/LV), ejection fraction (EF) and mortality. Pre-defined variables of interest were species, sex, age, weight, ischemia model (open vs closed and temporary vs permanent occlusion), occluded vessel, ischemia duration, follow-up duration, co-medication use, immunosuppression use and study quality.

Results

Our analyses yielded 246 relevant studies. Multivariable meta-regression revealed that IS/AAR was influenced independently by choice of species, sex, co-medication, occlusion type, occluded vessel, ischemia duration and follow-up duration. For IS/LV there were comparable results with occlusion type, occluded vessel and study quality all having a significant effect on the outcome. Multivariable meta-regression for EF measurements revealed species, sex and occlusion type as independent predictors of function after MI. Mortality analyses did not reveal any influence of the chosen variables.

Conclusion

We observed strong methodological variation in design of large animal MI studies. This study provides evidence that endpoints following MI in large animal studies significantly depend on study design and biological variation. Researchers should take into account the variability in these models to increase the rate of successful translation of new therapeutics in humans.

Abbreviations

MI	Myocardial infarction
IS/AAR	Infarct size as a ratio of the area at risk
IS/LV	Infarct size as a ratio of the left ventricle
EF	Left ventricular ejection Fraction
LCA	Left coronary artery
LCX	Left circumflex artery
LAD	Left anterior descending
RCA	Right coronary artery
CAMARADES	Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies

Introduction

Large animal studies are needed to test therapeutic efficacy of novel therapies for myocardial infarction (MI). These studies usually serve as crucial checkpoints before advancing to first-in-man trials.[1,2] Considerable heterogeneity exists in the models currently used to study MI and its aftermath.[3] The choice for a specific model may influence the manifestation and progression of the disease and subsequently the potential effect of an intervention or technique under evaluation.[3] There is a string demand for optimal selection of models that represent the human disease best, since many promising therapeutics have shown beneficial effects in the preclinical phases, but fail in the clinical setting.[4] Methodological flaws and inadequate modeling of human MI have been proposed as partial explanations of this 'translational failure', leading to false positive study outcomes and the risk of overestimation of effect size in preclinical studies.[5-8] Standardization of these animal models is crucial to value and compare individual studies to historical data, for which groups in the field of cardioprotection have put forth the first efforts.[2,9] Above all, the translational value of large animal MI models can be significantly increased if standardized models accurately resemble the disease under study.

In the evolving era of big data and abundant publication, the research community is calling on meta-research to systematically evaluate and improve research methods.[10,11] Systematic reviews and meta-analyses of preclinical data not only provide us with comprehensive overviews and bias assessments, but can also provide us with additional insights that explain heterogeneity within a specific disease and intervention.[12] In this perspective, combining and examining control groups of preclinical studies for a certain disease model, provides us with a comprehensive data-heavy method of studying the progression of the disease model and quantify the potential influence of certain variables on standard disease outcomes. The aim of the current study was to systematically explore the natural course of artificially induced MI in different large animal models and ultimately determine which biological and methodological factors act as effect modifiers, influencing disease course, primary endpoints and mortality within studies. Through meta-analysis, we report that functional and anatomical endpoints following MI in large animal models vary significantly due to variability in study design (Figure 1). Insights in determinants that explain this variability in outcome can be used to more closely resemble the clinical picture and thus to increase translational success of novel therapies, improve study quality and aid the standardization of MI models.

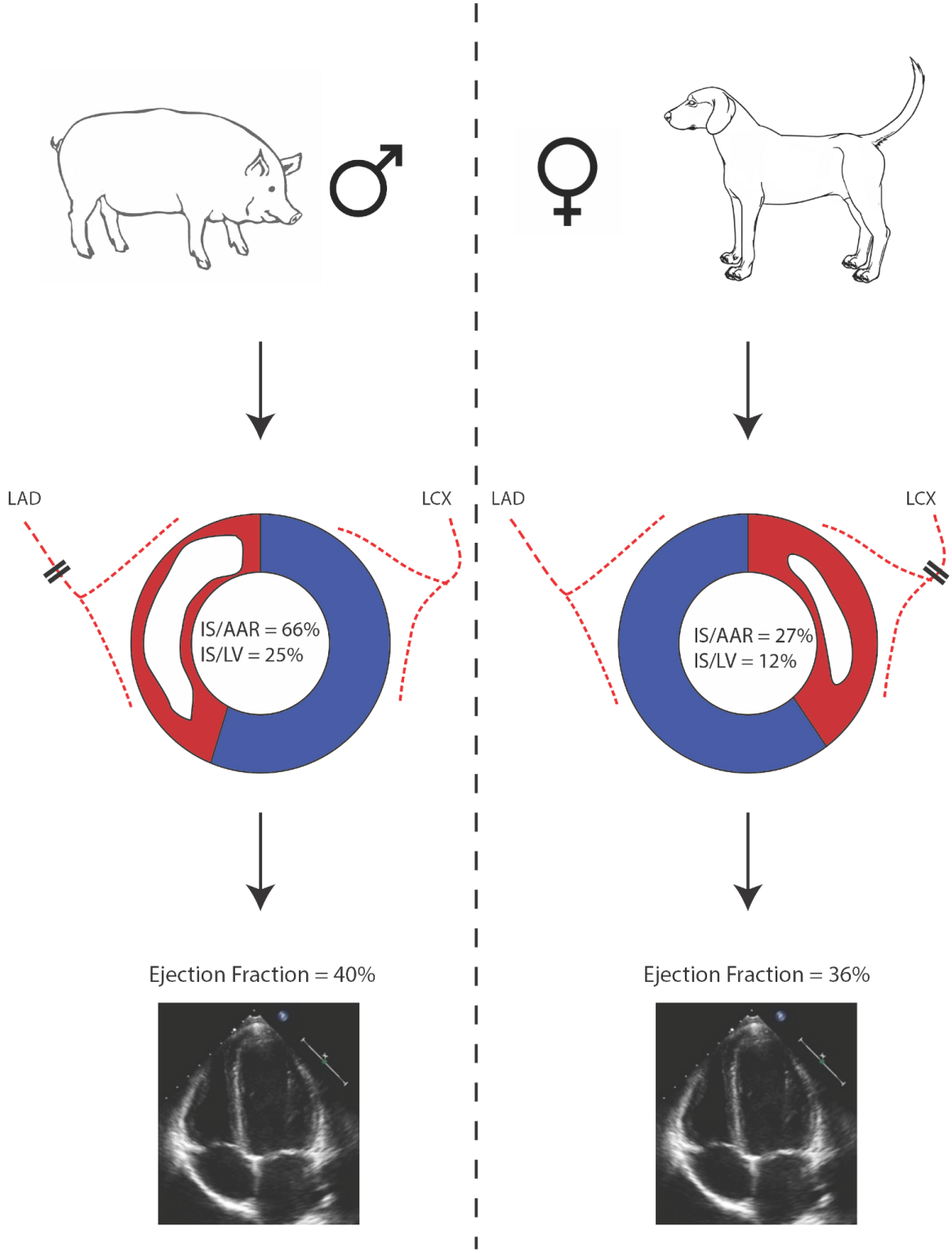

Figure 1. Graphical schematic representation of differences in outcomes after MI through study design; a model using male pigs and an LAD-occlusion will differ significantly from a female dog model with LCX-occlusion.

Methods

Data from control animals from two previous meta-analyses on large animal MI models were collected.[7,8] In both datasets infarct size as a ratio of the area at risk (IS/AAR), infarct size as a ratio of the left ventricle (IS/LV) and left ventricular ejection fraction (EF) were extracted and added in the current data if not present. Results on peri- and post-procedural mortality were extracted for all studies; peri-procedural meaning within the timeframe of the infarct-induction process ('death during surgical procedures') and post-procedural meaning after the disease-inducing procedure. Any procedural complications not due to the induction of the MI itself were not counted as 'natural' mortality. Due to evolving methodology over time in MI modeling with regards to the treatment of ventricular fibrillation (VF) during induction of MI, we recorded whether animals were treated for VF (either by medication or defibrillation) or were excluded immediately and performed a predefined sensitivity analysis to exclude a potential effect of this specific early exclusion. A thorough explanation of methodology on mortality data extraction can be found in the Supplementary section.

Pre-defined variables of interest were species, sex, age, weight, use of immunosuppression, co-medication commonly used in clinical care of MI (defined as being treated for the whole study after MI with one or more of the following compounds: aspirin, clopidogrel, ticagrelor, prasugrel, β-blockers, ACE-inhibitors, angiotensin receptor blockers, and/or statins), follow-up duration post-MI, study quality and multiple characteristics of the infarct induction procedure: open-thorax vs closed percutaneous procedure, permanent vs temporary occlusion, ischemia duration (if transient occlusion) and type of vessel occluded (left coronary artery (LCA) vs left circumflex artery (LCX) vs left anterior descending (LAD) vs right coronary artery (RCA)). Study quality was assessed using the 'Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies' (CAMARADES) quality checklist.[13] Any variable not already assessed prior to this project, was added to the database.

All data has been inserted in the CAMARADES database (available on request).[14]

Statistical analysis

Random effects meta-analysis with restricted maximum likelihood was performed due to anticipated heterogeneity between the different models of disease. Univariable meta-regression was performed for the association of chosen variables with our outcomes of interest. All variables were subsequently tested in multivariable meta-regression with the outcomes IS/AAR, IS/LV, EF and mortality, to correct for potential effect modification and to distinguish independent effects. Of note, multivariable meta-regression is especially suitable in the setting of animal studies, as all variables of interest are deliberately kept constant in preclinical study setup as opposed to the clinical setting. This minimalizes the risk of a potential ecological bias in our analysis. A post-hoc Wald test was used for categorical univariable meta-regression with more than two categories and in multivariable meta-regression to determine the individual association per individual variable. We used raw means for the outcomes IS/AAR, IS/LV and EF, since percentages are not expected to differ between the different groups under study.

For mortality outcomes, we used ratios (number of dead animals per total animals) and weighed each measurement on the inversed square root of the total number of animals for each comparison

in our meta-regression analysis ($1/\sqrt{n}$). In the case of two measurements in the same procedural setting (for example mentioning of mortality peri-procedural both before and after randomization), the appropriate ratio was determined by multiplying both proportions ($1\text{-}p_{total}=(1\text{-}p_1)*(1\text{-}p_2)$). The weighing factor for such a value is the square root of the total number of animals in both measurements, divided by two ($1/\sqrt{((n_1+n_2)/2)}$). A p-value of <0.05 was considered significant.
For our prediction modeling strategy, we used multivariable meta-regression to predict the outcomes for commonly used large animal models. We modeled both a pig and a dog model of temporary 60-minute occlusion with follow-up of 1 day, 1 week and 1 month. We did the same for a chronic occlusion pig model, using the same follow-up times. Statistical analyses were performed using R version 3.1.2[151528](28)(28)(28)[2828] with the additional metafor package[16] and Stata version 11 (Statacorp, LP, Texas, USA). The R script is available in the Supplementary section.

Results

A total of 246 studies were used, yielding 1500, 1221 and 775 animals for the outcomes IS/AAR, IS/LV and EF, respectively (Table 1). For the mortality analyses, data of 3622 animals and 1555 animals was studied for peri-procedural and post-procedural mortality, respectively (Table 1).

	Datasets			
	Jansen of Lorkeers et al.(7)	**van Hout et al.(8)**	**This meta-analysis**	**Average outcome (MA)**
IS/AAR	0	1500	1500	49.8%
IS/LV	261	960	1221	18.1%
Ejection Fraction	584	191	775	39.3%
Peri-procedural mortality	1183	2439	3622	16.7%
Post-procedural mortality	365	1190	1555	5.2%

Table 1. Number of included animals per dataset. MA=meta-analysis

Meta-analysis

From our datasets, an average IS/AAR of 49.8% (95%CI 46.0%-53.6%), IS/LV of 18.1% (95%CI 16.5%-19.7%) and EF of 39.3% (95%CI 37.4%-41.2%) were observed after MI induction and follow-up (Table 1). The average peri-procedural mortality and post-procedural mortality were 16.7% (95% CI 14.7%-18.7%) and 5.2% (95% CI 3.6%-6.9%) respectively (Table 1).

Meta-regression on standard outcomes: IS/AAR

Univariable meta-regression revealed multiple correlating variables with all our outcomes (Table 2-4), which were subsequently used for multivariable analyses.
Multivariable meta-regression (p<0.001) for the outcome IS/AAR revealed that **species** (-21% if dog compared to pig (p<0.001)), **sex** (-6% for both sexes compared to male (p=0.043) and -12% for unreported sexes compared to male (p=0.003)), **co-medication** (-15% if used (p=0.013)), **type of occlusion** (-33% if temporary compared to permanent occlusion (p<0.001) and -41% if temporary compared to unknown occlusion (p<0.001)), **occluded vessel** (+9% if LAD compared to LCX

(p=0.002)) and **follow-up duration** (-0.4% per hour of follow-up (p=0.001)) all independently influenced the outcome (Table 2). For all temporary occlusion studies (n=145), **ischemia duration** was an additional significant influencing variable in multivariable meta-regression of IS/AAR (+0.09%/min ischemia (p=0.001)) (Table 2).

Meta-regression on standard outcomes: IS/LV

Multivariable meta-regression analysis (p<0.001) for IS/LV showed that **occlusion type** (+4.1% if permanent compared to temporary (p<0.001)) and **occluded vessel** mattered (p=0.030). Furthermore, **study quality** was associated with a 1.3% difference in IS/LV per quality point (Table 3). The variable **sex** showed only a trend (p=0.061) for a potential association, with the same directions for categories as in the IS/AAR analyses (Table 3).

Meta-regression on standard outcomes: EF

Multivariable meta-regression for EF showed an effect of **species**, with a 7% difference in EF for pigs compared to sheep (p=0.03)). **Sex** also independently influenced EF after MI (-8% for female animals compared to studies using both sexes (p=0.006) and -7% for female animals compared to animals with unreported sex (p=0.025)) (Table 4). The choice of **occluded vessel** also showed an independent effect (+24.3 for only an LAD occlusion (p=0.016), +25.7 for only an LCX occlusion (p=0.013) compared to a combined LAD/LCX occlusion); this should be interpreted with caution, as the number of comparisons using either the LAD or LCX in the same study is limited (Table 4).

Mortality

Univariable meta-regression showed no variables investigated correlated with peri-procedural mortality (Table 5). The subsequent multivariable meta-regression was non-significant (p=0.33), so we did not proceed with further post-hoc testing. A sensitivity analysis, which omitted all animals that were excluded for VF with no attempt to treat the arrhythmia, was performed and also did not show any correlation with the variables of interest, both uni- and multivariably.

Univariable Analysis						Multivariable analysis			
Variable	categories	n	mean (95%CI)	p-value	post-hoc p-value	Variable	p-value	beta	post-hoc p-value
Species	Dog	122	46.4 (43.1-49.7)	**<0.001**	**0.001 (pig vs dog)**	Species	**<0.001**	**+21.0 if pig (vs dog)**	**<0.001**
	Pig	41	59.3 (53.6-65.0)		0.552 (pig vs sheep)			+6.9 if pig (vs sheep)	0.089
	Sheep	2	67.5 (41.0-93.9)		0.120 (dog vs sheep)			+1.6 if dog (vs sheep)	0.839
Sex	Male	45	47.7 (41.9-53.4)	0.80	0.718 (male vs female)	Sex	**0.029**	+10.5 if male (vs female)	0.113
	Female	11	50.0 (38.4-61.6)		0.681 (male vs unknown)			**+12.2 if male (vs unknown)**	**0.003**
	Both	78	51.3 (46.9-55.6)		0.718 (female vs unknown)			+1.7 if female (vs unknown)	0.768
	Unknown	31	49.5 (42.6-56.4)		0.321 (male vs both)			**+6.2 if male (vs both)**	**0.043**
					0.841 (female vs both)			-4.3 if female (vs both)	0.467
					0.668 (both vs unknown)			+4.3 if both (vs unknown)	0.467
Immunosupp				*not applicable*		Immunosupp		*not applicable*	
Comedication	yes	7	43.6 (29.2-58.0)	0.379		Comedication	**0.013**	-17.7 if used	
	no	158	50.2 (47.1-53.2)						
Open vs closed model	Open	129	50.3 (46.9-53.7)	0.536	0.293 (open vs closed)	Open vs closed model	0.141	+5.6 if open (vs closed)	0.080
	Closed	35	46.7 (40.2-53.3)		0.745 (open vs unknown)			-13.9 if open (vs unknown)	0.405
	Unknown	1	57.0 (18.7-95.3)		0.603 (closed vs unknown)			-19.6 if closed (vs unknown)	0.247
Occlusion	Permanent	17	69.1 (60.5-77.7)	**<0.001**	**<0.001 (permanent vs temporary)**	Occlusion	**<0.001**	**+32.7 if permanent (vs temporary)**	**<0.001**
	Temporary	145	47.2 (44.2-50.2)		0.072 (permanent vs unknown)			-8.3 if permanent (vs unknown)	0.397
	not known	3	65.9 (45.8-86.0)		0.774 (temporary vs unknown)			**-41.0 if temporary (vs unknown)**	**<0.001**
Occluded vessel	LAD	108	54.3 (50.8-57.7)	**<0.001**	**<0.001 (LAD vs LCX)**	Occluded vessel	**0.0023**	**+9.0 if LAD (vs LCX)**	**0.002**
	LCX	53	40.6 (35.6-45.6)		0.921 (LAD vs LAD/LCX)			+10.8 if LAD (vs LAD/LCX)	0.269
	LAD/LCX	4	55.2 (36.9-73.5)		0.13 (LCX vs LAD/LCX)			-8.8 if LCX (vs LAD/LCX)	0.291
Follow-up duration		165	-0.02/hour (-0.05-0.01)	0.12		Follow-up duration	**0.001**	**-0.04/hour**	
Study Quality		165	+0.36/point (-1.7-2.4)	0.734		Study quality	0.939	+0.08 / point	
Ischemia time		*145*	-0.01/min (-0.07-0.05)	0.723		Ischemia time**(n=56)*	**0.001**	**+0.09/min**	
Weight		*159*	+0.48/kg (0.219-0.743)	**<0.001**		Weight**(n=159)*	0.333	+0.15/kg	
Age		*5*	-0.25/wk (-3.24-2.74)	0.806		Age**(n=5)*		*not applicable*	

*variable was added to the multivariable model separately, due to missing data

Table 2. Univariable and multivariable meta-regression for outcome IS/AAR and chosen variables. Total multivariable meta-regression was significant (p<0.0001).
n= the number of comparisons (=165 in total)

Univariable Analysis						Multivariable analysis			
Variable	categories	n	mean (95%CI)	p-value	post-hoc p-value	Variable	p-value	beta	post-hoc p-value
Species	Dog	90	16.7 (14.9-18.5)	**0.040**	**0.015 (pig vs dog)**	Species	0.236	+4.3 if pig (vs dog)	0.101
	Pig	52	20.4 (18.0-22.8)		0.640 (pig vs sheep)			-2.1 if pig (vs sheep)	0.795
	Sheep	1	24.4 (7.7-41.1)		0.365 (dog vs sheep)			-6.4 if dog (vs sheep)	0.442
Sex	Male	35	19.6 (16.7-22.5)	0.247	0.733 (male vs female)	Sex	0.061	+5.4 if male (vs female)	0.108
	Female	18	18.7 (14.6-22.8)		0.746 (male vs unknown)			**+5.4 if male (vs unknown)**	**0.024**
	Both	50	16.0 (13.6-18.5)		0.930 (female vs unknown)			-0.01 if female (vs unknown)	0.995
	Unknown	40	18.9 (16.2-21.6)		0.069 (male vs both)			**+4.8 if male (vs both)**	**0.012**
					0.841 (female vs both)			-0.7 if female (vs both)	0.835
					0.120 (both vs unknown)			+0.6 if both (vs unknown)	0.757
Immunosupp	yes	3	12.1 (2.1-22.1)	0.236		Immunosupp	0.106	-8.0 if used	
	no	140	18.2 (16.7-19.7)						
Comedication	yes	9	15.4 (9.4-21.3)	0.361		Comedication	0.133	-5.0 if used	
	no	134	18.2 (16.7-19.7)						
Open vs closed model	Open	99	18.7 (16.9-20.4)	0.224		Open vs closed model	0.918	+0.2 if open model	
	Closed	44	16.7 (14.1-19.4)						
Occlusion	Permanent	46	20.2 (17.6-22.7)	0.138	0.047 (permanent vs temporary)	Occlusion	**0.033**	**+4.1 if permanent (vs temporary)**	**0.012**
	Temporary	95	17.0 (15.3-18.8)		0.677 (permanent vs unknown)			+0.5 if permanent (vs unknown)	0.932
	not known	2	17.6 (5.4-29.7)		0.933 (temporary vs unknown)			-3.8 if temporary (vs unknown)	0.529
Occluded vessel	LAD	93	19.2 (17.4-20.9)	**0.004**	0.130 (LAD vs LCX)	Occluded vessel	**0.030**	+1.1 if LAD (vs LCX)	0.515
	LCX	47	16.8 (14.4-19.3)		**0.002 (LAD vs LAD/LCX)**			**+13.1 if LAD (vs LAD/LCX)**	**0.009**
	LAD/LCX	3	3.7 (-5.7-13.1)		**0.008 (LCX vs LAD/LCX)**			**+12.0 if LCX (vs LAD/LCX)**	**0.018**
Follow-up duration		143	+0.001/hour (-0.001-0.002)	0.565		Follow-up duration	0.154	-0.002/hour	
Study Quality		143	+1.52/point (0.67-2.37)	**0.001**		Study quality	**0.033**	**+1.3 / point**	
Ischemia time		*95*	+0.002/min (-0.002-0.006)	0.414		Ischemia time**(n=95)*	0.143	+0.003/min	
Weight		*137*	-0.006/kg (-0.17-0.16)	0.946		Weight**(n=137)*	0.394	+-0.08/kg	
Age		*11*	+0.05/wk (-0.14-0.25)	0.568		Age**(n=11)*		*not applicable*	

*variable was added to the multivariable model separately, due to missing data

Table 3. Univariable and multivariable meta-regression for outcome IS/LV. Total multivariable meta-regression was significant (p=0.0003).
n= the number of comparisons (=143 in total)

Univariable Analysis						Multivariable analysis			
Variable	categories	n	mean (95%CI)	p-value	post-hoc p-value	Variable	p-value	beta	post-hoc p-value
Species	Dog	15	36.5 (31.3-41.8)	**0.011**	0.144 (pig vs dog)	Species	**0.035**	+5.3 if pig (vs dog)	0.10
	Pig	87	40.7 (38.6-42.8)		**0.005 (pig vs sheep)**			**+6.9 if pig (vs sheep)**	**0.03**
	Sheep	11	31.9 (26.1-37.7)		0.238 (dog vs sheep)			+1.6 if dog (vs sheep)	0.712
Sex	Male	21	37.9 (33.6-42.2)	0.068	0.398 (male vs female)	Sex	**0.035**	+4.6 if male (vs female)	0.12
	Female	27	35.4 (31.6-39.3)		0.206 (male vs unknown)			-2.0 if male (vs unknown)	0.453
	Both	15	39.4 (36.9-47.5)		0.018 (female vs unknown)			**-6.6 if female (vs unknown)**	**0.006**
	Unknown	50	41.2 (38.4-44.0)		0.214 (male vs both)			-2.9 if male (vs both)	0.413
					0.043 (female vs both)			**-7.5 if female (vs both)**	**0.025**
					0.741 (both vs unknown)			+1.6 if both (vs unknown)	0.758
Immunosupp	yes	6	37.4 (29.1-45.7)	0.658		Immunosupp	0.640	-2.2 if used	
	no	107	39.3 (37.1-41.5)						
Comedication	yes	11	43.7 (37.6-49.9)	0.135		Comedication	0.295	+3.5 if used	
	no	102	38.8 (36.8-40.8)						
Open vs closed model	Open	50	39.1 (36.2-42.0)	0.868		Open vs closed model	0.265	+2.3 if open model	
	Closed	63	39.4 (36.8-42.0)						
Occlusion	Permanent	56	36.5 (33.9-39.1)	**0.013**	**0.005 (permanent vs temporary)**	Occlusion	0.063	-4.5 if permanent (vs temporary)	0.036
	Temporary	55	41.9 (39.2-44.5)		0.175 (permanent vs unknown)			-10.0 if permanent (vs unknown)	0.187
	not known	2	46.5 (32.2-60.7)		0.531 (temporary vs unknown)			-5.5 if temporary (vs unknown)	0.469
Occluded vessel	LAD	89	41.2 (36.7-45.7)	**0.011**	0.618 (LAD vs LCX)	Occluded vessel	**0.045**	-0.7 if LAD (vs LCX)	0.568
	LCX	23	41.0 (32.4-49.6)		**0.003 (LAD vs LAD/LCX)**			**+24.3 if LAD (vs LAD/LCX)**	**0.016**
	LAD/LCX	1	10 (-8.1-28.1)		**0.003 (LCX vs LAD/LCX)**			**+25.7 if LCX (vs LAD/LCX)**	**0.013**
Follow-up duration		113	-0.0002/hour (0-0.0003)	0.338		Follow-up duration	0.12	-0.0004/hour	
Study Quality		113	0.14/point (-1.4-1.7)	0.859		Study quality	0.234	-1.0 / point	
Ischemia time		*55*	-0.04/min (-0.1-0.05)	0.416		Ischemia time**(n=55)*	0.740	0.016/min	
Weight		*98*	0.06/kg (-0.091-0.231)	0.428		Weight**(n=98)*	0.317	+0.09/kg	
Age		*24*	0.17/wk (-0.116-0.449)	0.234		Age**(n=24)*	0.810	-0.10/wk	

*variable was added to the multivariable model separately, due to missing data

Table 4. Univariable and multivariable meta-regression for outcome ejection fraction. Total multivariable meta-regression was significant (p=0.0012).
n= the number of comparisons (=113 in total)

Univariable Analysis							Multivariable analysis			
Variable	categories	% mortality peri-proc (n)	p-value	% mortality post-proc (n)	p-value	post-hoc p-value	Variable	p-value peri-proc	p-value post-proc	beta
Species	Dog	17.8% (93)	0.26	5.4% (122)	0.95		Species	NA	NA	
	Pig	14.6% (68)		5.1% (41)						
	Sheep	20.3% (9)		4.5% (2)						
Sex	Male	15.2% (63)	0.24	5.3% (39)	0.87		Sex	NA	NA	
	Female	13.7% (24)		5.% (25)						
	Both	19.8% (41)		5.9% (59)						
	Unknown	18.0% (41)		4.1% (30)						
Immunosupp	yes	*0% (1)*	*0.23*	0% (2)	0.44		Immunosupp	NA	NA	
	no	*16.8% (169)*		5.3% (152)						
Comedication	yes	10.9% (7)	0.27	5.7% (7)	0.90		Comedication	NA	NA	
	no	16.9% (163)		5.2% (147)						
Open vs closed model	Open	16.1% (118)		5.1% (105)	0.78		Open vs closed model	NA	NA	
	Closed	18.0% (52)		5.6% (49)						
Occlusion	Permanent	17.0% (43)	0.24	6.2% (39)	**0.005**	perm vs temp = 0.361	Occlusion	NA	NA	
	Temporary	16.9% (125)		4.6% (114)		**perm vs unknown = 0.003**				
	Unknown	0% (2)		34.8% (1)		**temp vs unknown = 0.002**				
Occluded vessel	LAD	16.8% (116)	0.72	4.8% (102)	0.72		Occluded vessel	NA	NA	
	LCX	15.9% (51)		6.4% (48)						
	LAD/LCX	26.9% (2)		3.7% (3)						
	Unknown	22.2% (1)		0% (1)						
Study Quality		-0.77/point (170)	0.28	-0.024/point (154)	0.96		Study Quality	NA	NA	
Follow-up duration		-0.004/hr (166)	0.78	0.0023/hr (152)	**0.03**		Follow-up duration (n=113)*	NA	**<0.001**	**0.007/hour**
Ischemia time		0.006/min (123)	0.10	-0.002/min (114)	0.52		Ischemia time *(n=113)**	NA	0.77	-0.0007/min
Weight		-0.1/kg (153)	0.29	-0.06/kg (138)	0.30		Weight*(n=153)**	NA	NA	
Age		+0.06/wk (15)	0.79	-0.015/wk (12)	0.92		Age*(n=15)**	NA	NA	

*variables added to the multivariable model separately, due to missing data

Table 5. Univariable and multivariable meta-regression for peri- and post-procedural mortality.
Multivariable meta-regression was not significant (p=0.33 and p=0.42). Multivariable meta-regression with the addition of ischemia time was significant for post-procedural mortality (p=0.04). n= the number of comparisons (=170 and 165 in total)

Univariable meta-regression for post-procedural mortality showed a correlation with follow-up time, with the addition of 0.002% per hour extra follow-up (p=0.03). Multivariably, meta-regression was not significant and no further post-hoc analyses were done (p=0.41). The selected multivariable regression with the addition of ischemia duration (which only applies to temporary occlusion models) was significant (p= 0.047) and post-hoc testing revealed follow-up time as the only significant independent predictor of post-procedural mortality (0.007%/hour, p=0.001) in studies using a temporary occlusion model.

Prediction of outcomes in common large animal MI models

Predicted outcomes for predefined commonly used models were generated (Table 6), showing clear differences for all outcomes between these models.

Pig I/R (60 min) LAD model

	Infarct size / Area at Risk	Infarct size / Left Ventricle	Ejection Fraction
1 day	~60%	~18%	-^
1 week	~55%	~18%	~43%
4 weeks	(~37%)*	~18%	~43%

Dog I/R (60min) LAD model

	Infarct size / Area at Risk	Infarct size / Left Ventricle	Ejection Fraction
1 day	~40%	~15%	-^
1 week	~35%	~15%	~36%
4 weeks	(~18%)*	~14%	~36%

Pig permanent LAD model

	Infarct size / Area at Risk	Infarct size / Left Ventricle	Ejection Fraction
1 day	~88%	~24%	-^
1 week	~82%	~24%	~38%
4 weeks	(~60%)*	~23%	~38%

Table 6. Predicted regular outcomes for common large animal MI models. assuming linear effect of follow-up duration. ^not calculated due to few measurements and myocardial stunning

Discussion

The current meta-analysis systematically reveals the effect of methodological choices on primary outcome measurements in large animal MI studies. The identification of the effect of the different experimental setups is of great importance, since it will guide adequate expectations of study results and mortality for specific models. It also enables more adequate and precise power calculations, which are essential when designing any preclinical study. We can now quantify biological differentiating variables for certain effect sizes and more accurately determine if these models resemble human disease. We confirmed some known biological variability within these

models, showed effects that can be translated to the human situation and were able to quantify these variations in a meta-analytic manner.

Identified effect modifiers for standard outcome measures

The different disease manifestation across **species** has been demonstrated in the past[17], with canine hearts forming more collaterals than hearts of other species, which we broke down to a ~20% smaller IS/AAR for dog models compared to pig models and lower EF in sheep compared to pigs. Conserved within evolution, females seem to show smaller infarcts compared to mixed groups and male counterparts, which is in line with the clinical data on **sex influence** on infarct size, favoring female subjects.[18-20] Of note, using female animals might leave researchers with a smaller therapeutic window in infarct size, potentially explaining the reduced efficacy of anti-inflammatory compounds in female animals.[8] Interestingly, pump function seems more decreased in female animals, once again arguing that the different sexes do not respond completely similar to cardiac damage and subsequent remodeling. In this perspective, it is crucial for translational success to include both sexes in future preclinical research, as is also called for by the NIH in preclinical projects.[21] Furthermore, there seem to be fewer studies using (only) female animals in our dataset, potentially explaining why not all comparisons to the female group always reached statistical significance.

The observed difference of ~9% in IS/AAR for **different occlusion sites** (LAD vs LCX) is in line with the observed greater loss of regional systolic function for anterior wall ischemia[22], but was not observed for the outcome EF.

The observed reduction of infarct size and EF when increasing **follow-up time** is interesting both from a methodological and biological point of view. Smaller infarct sizes might imply smaller therapeutic windows for new interventions, while a larger reduction in EF might account for the inverse reasoning. Biologically this might be explained by infarct resorption and subsequent myocardial wall thinning, resulting in a decreased attribution of the thinned scar to the total myocardial mass.[23] Other explanations could be possible regeneration and post-infarction hypertrophy. Hibernating myocardium is not likely to explain this phenomenon, as function should increase after myocardial stunning and hibernation in the early stages of an infarct. Regardless of the cause, a longer follow-up could lead to more clinically relevant conclusions and might need more power to show any true differences. Incorporation of regular MI **co-medication** also seems to reduce the IS/AAR, which might be crucial for clinically relevant translation to the same poly-pharmaceutical human situation. A limitation of this variable is of course bundling of all studies using one or more of these compounds for power-reasons; we are not able to pinpoint these effects to one single compound. However, for many of these compounds there is either preclinical or even clinical evidence that they can influence infarct size and other outcomes after MI and therefore might be relevant to take into account for future experimental study design.[24,25]

Interestingly, the composition of the dataset blurred the effect of multiple variables in the univariable analysis for IS/AAR, while our multivariable approach revealed certain effects that would otherwise have gone unnoticed.

No difference in outcome was observed for **open versus closed modeling of MI**, in contrast to what has been demonstrated in a recent study.[26] This might mean that conclusions from certain

experiments can only be applied to the same setting; in this case an ischemia-reperfusion pig model. On the other hand, it might imply that meta-analyses cannot reveal all subtle differences within MI animal models. The same holds true for other variables in our dataset, like **immunosuppression**, which theoretically could have an effect on all our outcomes of interest.
Furthermore, we are limited by the data we were able to extract. In preclinical meta-analyses, many 'known unknowns' are present; variables that one would like to analyze, but are not reported as such. This is resembled by the unexplained heterogeneity (for multivariable IS/AAR analysis R^2= ~46% and I^2=~96%) that, in the case of our MI analyses, is potentially influenced by for example the specific occlusion site of the vessel (which directly influences the area at risk), weight of the animal or experience of the surgeon. However, with the variables available, we were able to explain a significant part of the observed heterogeneity, with model-specific differences and human-like variability for sex and co-medication.

Mortality
Modeling mortality in our study did not result in many explanatory variables, so we can only give summary estimates based on the meta-analysis of the total data. On average, peri-procedural mortality was ~17%, while post-procedural mortality was condensed in a ~5% mortality rate. These are important numbers for future study designs, as power analyses are crucial in the success chance of (pre)clinical trials and the reduction of both type I and type II errors. It is possible that these numbers are incomplete or biased in the current analysis, due to incomplete reporting in prior studies. This might be less of a problem for future similar analyses as the reporting of animal studies will hopefully improve substantially due to the ARRIVE guidelines, EDA application and journals demanding complete reporting.[27,28]

The need for meta-research on methods and reproducibility has been solicited for by the community and is a crucial process in the self-cleansing ability of research.[10] This paper untangled a part of the variation observed and generates realistic starting points for well-needed large animal MI models, hopefully adding further insight in disease understanding, accurate modeling of MI and more translational success for new cardiac interventions.
Being able to explain and predict a 'point of departure' in large animal MI models will prove useful to tailor experiments and make reasonable power calculations based on the expected damage, mortality and potential experimental effect (example in Figure 1). This will potentially result in more accurately powered studies, more definite answers to research questions and less waste of animal lives and research money.[29] Many clinically relevant patient characteristics seem to be of influence in the preclinical setting, and will potentially influence any outcome if not taken into account. In the current era of translational science, all researchers need to take this variation into account when designing new studies to optimize the chance of success of any large animal experiment.

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Standardized mean differences cause funnel plot distortion in publication bias assessments: the plot thickens

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ABSTRACT

Introduction

As the use of meta-analysis as a tool for the synthesis of (pre)clinical evidence continues to increase and diversify, there is a growing need for methodological research into optimal analysis. Meta-analyses often include an assessment of publication bias, based on asymmetry testing of funnel plots in which the effect size is plotted against the standard error (SE). Here, we show that funnel plots using the standardized mean difference (SMD) plotted against the SE are susceptible to distortion and misinterpretation; and we investigate alternative approaches.

Methods

We use existing meta-analyses to illustrate SMD funnel plot distortion, and use data simulation to assess the influence of the number of primary studies included, the presence or absence of an intervention effect, and the sample size of the primary studies, on the severity of funnel plot distortion in biased and unbiased meta-analyses. We also investigate the potential of using a sample size-based precision estimate, or using the Normalized Mean Difference (NMD), as alternative approaches in both simulated and empirical data.

Results

Converting the raw mean difference to SMD resulted in significant overestimation of funnel plot asymmetry by both Egger's regression and trim and fill analysis for two published preclinical meta-analyses. In simulated unbiased meta-analyses, publication bias as assessed by Egger's regression was systematically overestimated in SMD-SE funnel plots. Distortion was more severe when the primary studies had a small sample size, and when an intervention effect was present. In biased simulations, there was clear distortion of SMD-SE funnel plots, but not of funnel plots in which the SMD was plotted against a precision estimate based on the study sample size ($1/\sqrt{n}$), or funnel plots of the NMD plotted against the SE.

Conclusion – Although commonly reported, funnel plots using the SMD in combination with the SE are unsuitable for publication bias assessments and can lead to false-positive results, especially when small sample sizes are small (*e.g.* in preclinical studies). We propose using the NMD (when possible), or the SMD plotted against a precision estimate based on the sample size, as more reliable alternatives.

Keywords: publication bias, standardized mean difference, funnel plot, meta-analyses

Abbreviations

RMD	Raw Mean Difference
SMD	Standardized Mean Difference
NMD	Normalized Mean Difference
SD	Standard Deviation
SE	Standard Error
ES	Effect Size
n	Sample size
eq.	Equation
ctrl	Control
int	Intervention

Introduction

Systematic reviews are literature reviews intended to answer a particular research question by identifying, appraising and synthesizing all research evidence relevant to that question. They may include a meta-analysis, a statistical analysis in which outcome data from individual studies is combined, and can be used to estimate the direction and magnitude of any underlying intervention effect, and to explore sources of between-study heterogeneity. Simultaneously, meta-analysis is used to assess the risk of publication bias: the phenomenon that published research is more likely to have positive or statistically significant results than unpublished experiments.[1] Meta-analyses are routinely used in clinical research to guide clinical practice and healthcare policy, reduce research waste and increase patient safety.[2] The use of meta-analysis continues to increase[3] and it has become more common to apply these approaches to the synthesis of preclinical evidence.[4] This development calls for 1) methodological research to ensure that approaches (or: methods) to analysis are appropriate to data types (*e.g.* clinical *versus* preclinical) with particular characteristics; and 2) resources that guide and inform researchers, reviewers and readers involved in meta-analysis on best practice. In this light, here we present our findings on how the use of funnel plots based on the standardized mean difference (SMD) can introduce a risk of incorrect assessment of publication bias, particularly in meta-analyses of preclinical data characterised by a large number of individually small studies where there is substantial between-study heterogeneity.

Formulation of raw mean difference, standardized mean difference and normalized mean difference

In order to statistically combine data on for example the difference between two treatment arms from several studies, outcome measures are recalculated into a common intervention effect estimate. These include (for binary outcomes) the risk or odds ratios; and for continuous data a raw mean difference (RMD), SMD or normalized (or proportional) mean difference (NMD).

The RMD can be used when all outcome data are in the same measurement unit, and the interpretation of the outcome is the same in all settings (*i.e.* a certain change in outcome x has the same meaning in all studies). The RMD is calculated by subtracting the mean outcome value in the control group (M_{ctrl}) from the mean in the intervention group (M_{int}):

$$RMD = M_{int} - M_{ctrl} \ . \qquad (1)$$

When assuming that the standard deviation (SD) may differ between the experimental groups, the standard error (SE) of the RMD is calculated as:

$$SE_{RMD} = \sqrt{\frac{SD_{int}^2}{n_{int}} + \frac{SD_{ctrl}^2}{n_{ctrl}}}, \qquad (2)$$

where n is the sample size per group.

In cases where the measurement unit and/or the interpretation of the outcome differ between studies (*e.g.* a certain change in infarct size measured in mm^3 has a different meaning in the mouse

brain than in the rat brain), the intervention effect may be expressed as an SMD. Per study, the SMD is obtained by dividing the RMD by that study's pooled standard deviation (SD_{pooled}) to create an effect estimate that is comparable across studies:

$$SMD = d = \frac{M_{int} - M_{ctrl}}{SD_{pooled}} \quad (4)$$

, where SD_{pooled} is:

$$SD_{pooled} = \sqrt{\frac{(n_{ctrl}-1)\,SD_{ctrl}^2 + (n_{int}-1)\,SD_{int}^2}{n_{ctrl} + n_{int} - 2}} \quad (5)$$

Thus, the SMD expresses the intervention effect in all studies in the same new unit: the SD.
Per study, the standard error (SE) can be approximated using the sample sizes (n) and the effect estimate (SMD):

$$SE_{SMD} = \sqrt{\frac{(n_{ctrl}+n_{int})}{n_{ctrl} * n_{int}} + \frac{SMD^2}{2*(n_{ctrl}+n_{int})}} \quad (6)$$

Of note, the SMD can be estimated in two slightly different ways, depending on whether Cohen's d[5] (as in eq. 4 and 6), or Hedges' g[6] is used. (see supplemental material or reference[6] for full equations). One weakness of the SMD approach is that it rests on an assumption that the observed variance provides a close approximation to the population variance; and when sample size is small, as is often the case in animal studies, this assumption may not hold.
This gave rise to the development of a further estimate of effect size, the so-called "normalised mean difference". This does not involve normalisation in the statistical sense, but rather expresses the change as a proportion of the size of the lesion size observed in the control group. Per study, the NMD is calculated as:

$$NMD = 100\% \times \frac{(M_{int} - M_{sham}) - (M_{ctrl} - M_{sham})}{(M_{ctrl} - M_{sham})} \quad (7)$$

where M_{sham} is the mean score for normal, unlesioned and untreated subjects. The corresponding SE is calculated as:

$$SE_{NMD} = \sqrt{\frac{(100*\frac{SD_{ctrl}}{M_{ctrl}-M_{sham}})^2}{n_{ctrl}} + \frac{(100*\frac{SD_{int}}{M_{int}-M_{sham}})^2}{n_{int}}} \quad (8)$$

In many cases "normal" animals do not have a lesion, and the calculations simplify to

$$NMD = 100\% \times \frac{(M_{int}) - (M_{ctrl})}{(M_{ctrl})} \quad (6a)$$

$$SE_{NMD} = \sqrt{\frac{(100*\frac{SD_{ctrl}}{M_{ctrl}})^2}{n_{ctrl}} + \frac{(100*\frac{SD_{int}}{M_{int}})^2}{n_{int}}} \quad (8a)$$

(see[7] for a comprehensive overview of (preclinical) meta-analysis methodology).

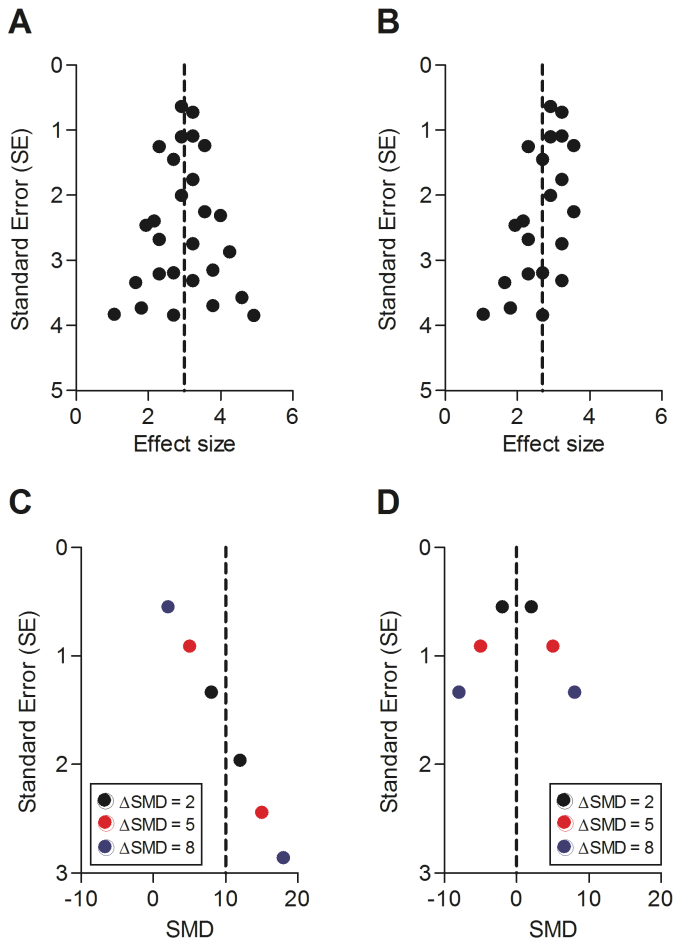
Note that equation 6 dictates that the SE_{SMD} is inherently correlated to the size of the SMD, whereas the SEs of the RMD (equation 2) and NMD (equation 8) are independent of the corresponding effect sizes.

Testing for publication bias

Assessment of publication bias frequently relies on an evaluation of funnel plot (a)symmetry. Funnel plots are scatter plots of the effect sizes of the included studies *versus* a measure of their precision, usually the SE or 1/SE. In the absence of bias and heterogeneity, funnel plots should be funnel-shaped and symmetrically centred around the summary effect estimate of the analysis, since 1) imprecise (smaller) studies will deviate further from the summary effect compared to precise (larger) studies and 2) studies are equally likely to overestimate or underestimate the true effect. In case of publication bias, studies showing small, neutral or controversial effects are more likely to remain unpublished. As a result, the funnel plot will become asymmetrical, and the summary effect estimate will shift accordingly (Boxed Figure 1A and B).

As shown above, the SE_{SMD} of a study is correlated to the SMD (see eq. 4 and 6): the larger the SMD, the larger the associated SE. Because of this correlation, a funnel plot using both parameters might become asymmetrical in the absence of publication bias. Thus, when funnel plot distortion is assessed by visual inspection, this skewing might cause the plot to be interpreted as being asymmetrical and lead the observer to erroneously conclude that publication bias is present. Funnel plot asymmetry is often tested statistically using Egger's regression[8] or Duval and Tweedie's trim and fill analysis.[9] However, to our knowledge these methods have been not been validated for use with the SMD, or in small study settings. Neither of these analyses take skewing of SMD funnel plots into account, which may lead to incorrect findings regarding publication bias.

Here, we investigate the reliability of RMD, SMD and NMD-based funnel plots for the assessment of publication bias in meta-analyses, using both empirical datasets and data simulations. We investigate the effect of the study sample size, the number of studies in the meta-analysis and the magnitude of the intervention effect on the severity of funnel plot distortion. We assess whether distortion can be avoided by using a precision estimate based on the sample size of the primary studies, as previously suggested for mean difference outcome measurements.[10] We then use this alternative approach to reanalyse published funnel plots, and show that these systematic reviews may have overestimated the severity of publication bias in their body of evidence. Our findings have important implications for the meta-research field, since authors may have reached incorrect conclusions regarding the existence of publication bias based on funnel plots using the SMD measure of effect size.



Box 1: **A-B**: hypothetical funnel plots in the absence (**A**) and presence (**B**) of bias. The precision estimate used is the standard error (SE). Dashed lines indicate the summary effect estimate. **C-D**: funnel plots of data expressed as standardized mean difference (SMD) with a summary effect estimate of 10 (**C**) or 0 (**D**). ΔSMD = the deviation of the individual study SMD from the summary SMD. Given equal sample sizes, studies with the same deviation from the summary effect (*e.g.* SMDs of 5 and 15, ΔSMD = 5 in panel **C**) will not have the same absolute value for SMD^2 in the equation of the SE ($5^2 \neq 15^2$) (eq. 6), and thus will not have the same SE. This will cause funnel plot distortion, since studies with a relatively small effect size (and associated SE) skew towards the upper left region of the plot. Simultaneously, studies with a relatively large effect size skew towards the bottom right region of the plot, as the associated SE of these studies will be relatively large. When the summary effect is 0 (panel **D**), data points with the same deviation from the summary effect (*e.g.* ΔSMD = 5) will have the same absolute value for SMD^2 (*e.g.* 5^2 or -5^2) in the equation of the SE, and will therefore have the same precision estimate. Under these circumstances the points are therefore symmetrically distributed around the summary effect estimate.

Methods

For the re-analysis of empirical data we used two systematic reviews assessing the efficacy of ischaemic preconditioning[11] and stem cell treatments[12] on the outcome following myocardial ischaemia. We performed data simulations and re-analyses of empirical data using R statistical software (version 3.1.2) and the most recent MBESS, xlsx, meta and metafor packages (see Supplemental file 1 for R scripts).[13-17] For all analyses involving RMD and SMD the primary outcome of interest was the number of asymmetrical funnel plots as detected by Egger's regression.[8] As a secondary outcome, we assessed the number of missing studies as imputed by

Duval and Tweedie's trim and fill analysis[9] using a random-effects model, for simulation 1 (see below). We used trim and fill analysis in R to seek evidence for publication bias overstating the effectiveness of the interventions, based on the proposed direction of the intervention effect. We used Hedges' g to estimate pooled variance for SMD effect sizes, but also carried out sensitivity analyses using Cohen's *d* instead. We considered a p-value of <0.05 to be significant for Egger's regression in individual simulations. When testing the differences between the approaches (RMD, SMD and NMD) we used the Bonferroni correction to adjust the p-value for multiple comparisons.

For our empirical data from published preclinical meta-analyses[11,12] we constructed funnel plots using the unbiased SMD (Hedges' g[6]) and SE and compared these to funnel plots using the RMD and SE (as in the original publication).

Data simulations

In our first simulation, we tested the estimation of publication bias using SMD in simulated data where there was no publication bias. The pre-specified starting values for simulation data are shown in table 1. In brief, we simulated controlled studies by randomly sampling individual subject data from a normal distribution with a mean and SD belonging to either a control group or an intervention group. The selected means and SDs were based on outcome data for functional imaging in myocardial infarction studies (see table 1). To assess the influence of the presence *versus* absence of an intervention effect, the intervention group was sampled from either the same normal distribution as the control group, or one where a treatment effect had been introduced (table 1). The individual subject data were then aggregated into group means and variances for individual studies. To assess the effect of the study sample size on funnel plot distortion, we simulated two types of study sizes: small, in which we used typical sample sizes used in *e.g.* experimental myocardial infarction[11,12] and stroke[18,19]), and large, in which the sample sizes ranged between 60 and 320, as often used in clinical trials. For each simulated study, we determined the number of subjects by sampling the group sizes from the uniform distribution within the ranges of study sizes given.

Simulation and aggregation of individual subject data into study-level data was repeated until the desired number of studies to be included in the meta-analysis was obtained. We assessed the influence of the number of included studies on funnel plot distortion by simulating meta-analyses containing either 30, 300, or 3000 studies (N.B. 3000 studies are not considered a realistic number, but are simulated for illustrative purposes). Importantly, there was no publication bias introduced in these datasets and the funnel plots should therefore be symmetrical. We simulated 1000 meta-analyses for all scenarios and expressed the study results as RMD or SMD, in funnel plots with the effects size plotted on the x-axis and the SE as precision estimate plotted on the y-axis (RMD-SE and SMD-SE plots).

Experimental groups	Small studies			Large studies		
	n	*mean*	*SD*	*n*	*mean*	*SD*
Intervention 1 (no effect)	7-14	30	10	40-150	30	10
Intervention 2 (effect)	7-14	40	10	40-150	40	10
Control	5-16*	30	10	20-170*	30	10
Sham	4-6	70	4			

Table 1. Simulation characteristics. n = sample size; ND = normal distribution; SD = standard deviation; *control group sample size = intervention group sample size ±≤2 (small studies) or ±≤20 (large studies). The true treatment effect for Intervention 1 is 0 and for intervention 2 is 1 (SMD), 10 (RMD) or 0.25 (NMD).

Informed by the outcomes of simulation 1, in our second simulation we selected the conditions introducing the most prominent distortion in SMD-SE funnel plots to investigate the performance of alternatives including SMD-$1/\sqrt{n}$ funnel plots and NMD funnel plots. Thus, all simulations were performed with a small study sample size, in the presence of an intervention effect (see table 1) and with 3000 studies per meta-analysis. Under these conditions, we constructed RMD-SE and SMD-SE funnel plots as described above, as well as funnel plots of the SMD against the inversed square root of the total sample size ($1/\sqrt{n}$) in each study, and of the NMD against the SE. For the NMD, sham group data were simulated to have a mean of 70 and an SD of 4 (table 1). Group size was selected to be 4-6 subjects, which is a typical sample size for sham groups in preclinical experiments. We performed the simulations once and compared outcomes across all four funnel plots.

In our final simulation, we investigated the effects of a modelled publication bias on the performance of the SMD-SE and alternative approaches. We simulated meta-analyses containing 300 studies with a small sample size and a treatment effect present ($\Delta\mu$ = difference in means between control and intervention group = 10; see table 1). RMD-SE, RMD-$1/\sqrt{n}$, SMD-SE, SMD-$1/\sqrt{n}$ and NMD-SE funnel plots were constructed and tested for asymmetry using Egger's regression. We then introduced publication bias in these meta-analyses by removing all studies in which the difference between the intervention and control group means resulted in $p \geq 0.10$ in a two-sided Student's t-test. Funnel plot asymmetry testing was performed as above, and the results were compared to the unbiased simulations and between different funnel plot types. All simulations were repeated 1000 times. Of note, this simulation was not performed for meta-analyses of studies with a large sample size, since pilot data showed that the large sample size will cause only very few studies to be removed from the "biased" meta-analysis.

Finally, to assess the usefulness and impact of using a sample size-based precision estimate in SMD funnel plots of empirical data, we re-analysed data from five published preclinical meta-analyses that used SMD-SE funnel plots to assess publication bias. For these data sets, we compared the outcomes of Egger's regression and trim and fill analysis when using SMD-SE funnel plots *versus* SMD-$1/\sqrt{n}$ funnel plots. We obtained the corresponding author's consent for reanalysis.

Results

Publication bias assessment using RMD *versus* SMD funnel plots of two preclinical RMD datasets

Dataset 1 (ischaemic preconditioning) contains 785 data points.[11] In the original analysis using the RMD as effect measure, funnel plot asymmetry was detected by Egger's regression ($p=1.7x10^{-5}$), but no additional studies were imputed in trim and fill analysis (Figure 1A). When expressing the same data as SMD, funnel plot asymmetry increased dramatically (Figure 1B; $p<1.0x10^{-15}$) and 196 missing studies were imputed by trim and fill analysis, leading to a reduction of the SMD effect estimate from 2.8 to 1.9.

Dataset 2 (stem cell treatments) contained 95 data points.[12] Funnel plot asymmetry was detected in the original analysis using RMD (p=0.02) and trim and fill analysis suggested a reduction in effect

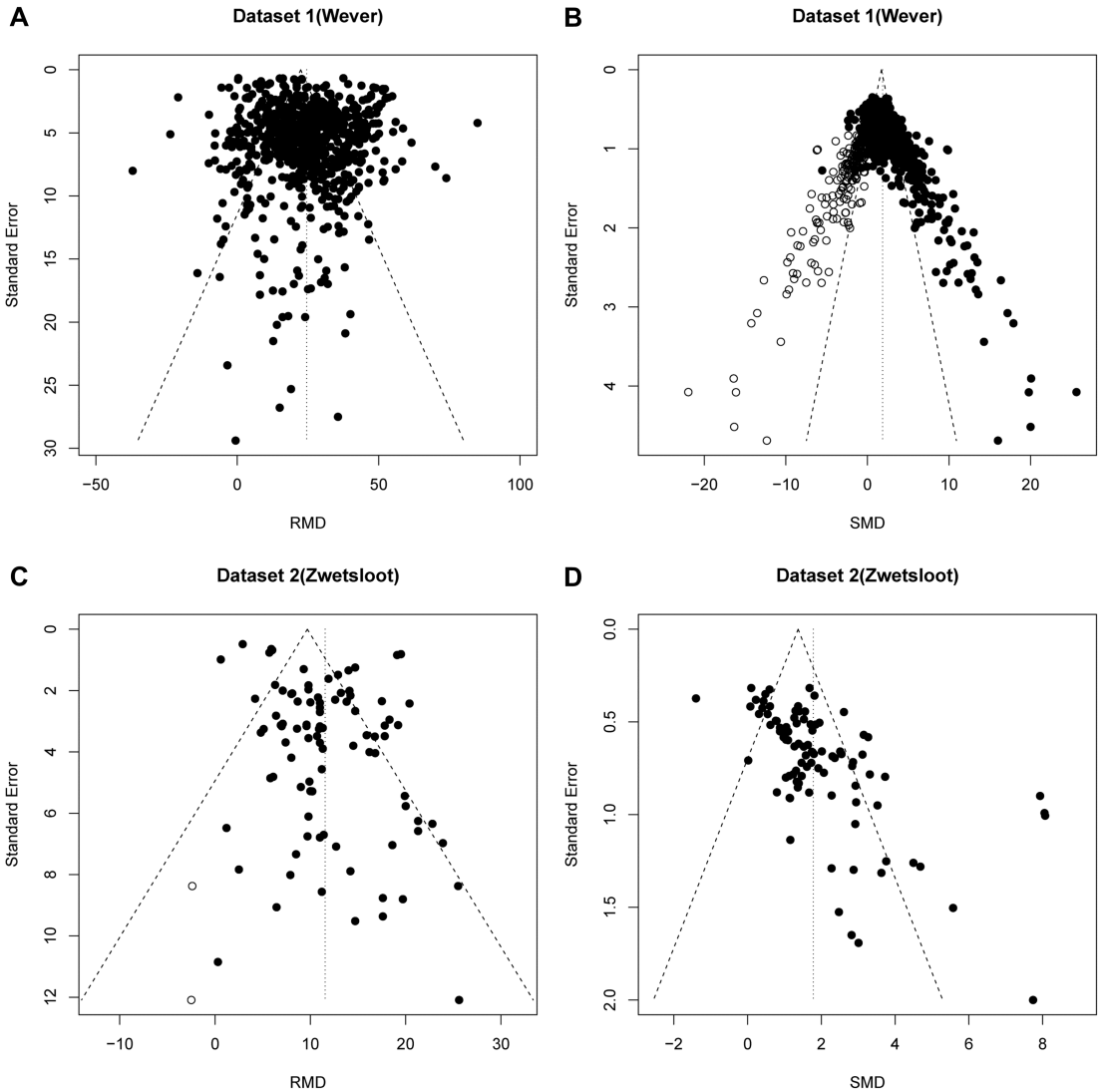


Figure 1. practical examples of funnel plot distortion, when plotting raw mean difference (RMD) data as standardized mean difference (SMD) for preclinical dataset 1 (A-B) and dataset 2 (C-D). Filled circles = observed data points; open circles = missing data points as suggested by trim and fill analysis.

estimate of 0.1% (from x to y) after filling two additional studies (Figure 1C). In contrast, a funnel plot of the same data expressed as SMD showed increased significant asymmetry ($p=3.4x10^{-10}$), but no missing studies to be imputed (Figure 1D).

Publication bias assessment using RMD *versus* SMD funnel plots through simulations

Results of our first simulation (in the absence of publication bias) are shown in Table 2, and exemplary funnel plots of these simulations in Figure 2 (small study sample size) and Supplemental Figure 1 (large study sample size). When we simulated no intervention effect, neither Egger's regression nor trim and fill analysis gave different results for the RMD-SE and SMD-SE analyses (Table 2, Figure 2A, B, E and F and Supplemental Figure 1A, B, E and F) and in ~95% of cases there was no evidence of asymmetry. Most simulated funnel plots were assessed as symmetrical, however in 5% of the cases the funnel plot appeared asymmetrical by chance.

When we simulated the presence of an intervention effect ($\Delta\mu = 10$; RMD = 10 and SMD = 1), ≥94% of the RMD funnel plot analyses were judged symmetrical (Table 2, Figure 2C and G, and Supplemental Figure 1C and G). In contrast, when using the SMD, funnel plot asymmetry was detected in at least 60% of the simulated funnel plots of where the size of contributing studies was small (Figure 2D and H and Supplemental Figure 1D and H), increasing as the number of individual studies contributing to the meta-analysis increased. When the size of contributing studies increased to 60-320 subjects, respectively 9%, 34% and 100% of the SMD funnel plots with 30, 300 or 3000 studies were assessed as asymmetrical (Table 2, Supplemental Figure 1). Through visual inspection, distortion could be distinguished in all SMD funnel plots that incorporated a true effect, most prominent in the preclinical (small study) scenarios (Figure 2 & Supplemental Figure 1).

Next, we tested the performance of SMD-$1/\sqrt{n}$ funnel plots and NMD funnel plots in the presence of an intervention effect as alternatives to the SMD-SE funnel plot. As in simulation 1, distortion of the SMD-SE funnel plot (Figure 3A) was again observed when compared to the RMD-SE plot (Figure 3B). However, when the precision estimate was changed from SE to $1/\sqrt{n}$ this distortion was not seen with either SMD or RMD (Figure 3D). Using NMD produced an undistorted funnel plot under all approaches (Figure 3C).

Study n	Number of studies in meta-analysis	Δμ	% of simulations witl Egger's p<0.05	Number of filled studies (mean±SD)
12-30	30	0	RMD: 3.6	RMD: 2.1±2.6
			SMD: 5.8	SMD: 1.6±2.3
	30	10	RMD: 6.0	RMD: 2.0±2.5
			SMD: 59.8	SMD: 4.1±2.9
	300	0	RMD: 3.6	RMD: 25.1±20
			SMD: 7.8	SMD: 19.2±18.1
	300	10	RMD: 5.9	RMD: 25.2±20.3
			SMD: 100.0	SMD: 61.0±5.1
	3000	0	RMD: 4.7	RMD: 257.7±166.2
			SMD: 7.9	SMD: 191.1±147.2
	3000	10	RMD: 4.3	RMD: 244.5±171.1
			SMD: 100.0	SMD: 616.5±16.1
60-320	30	0	RMD: 6.8	RMD: 2.4±2.7
			SMD: 6.8	SMD: 2.3±2.7
	30	10	RMD: 5.0	RMD: 2.4±2.7
			SMD: 8.7	SMD: 2.4±2.7
	300	0	RMD: 5.7	RMD: 19.0±16.3
			SMD: 5.9	SMD: 18.8 ±16.2
	300	10	RMD: 4.9	RMD: 18.9±16.3
			SMD: 33.6	SMD: 28.1±16.8
	3000	0	RMD: 5.1	RMD: 136.3±97.2
			SMD: 5.1	SMD: 133.6±94.6
	3000	10	RMD: 4.0	RMD: 138.9±98.7
			SMD: 99.8	SMD: 333.4±47.6

Table 2. Study characteristics in relation to publication bias assessment in simulation of unbiased meta-analyses (simulation 1). n= total subjects per study; Δμ= difference in normally distributed means between intervention and control group; MA = meta-analysis; RMD = raw mean difference; SMD = standardized mean difference; sims = simulations; SD = standard deviation.

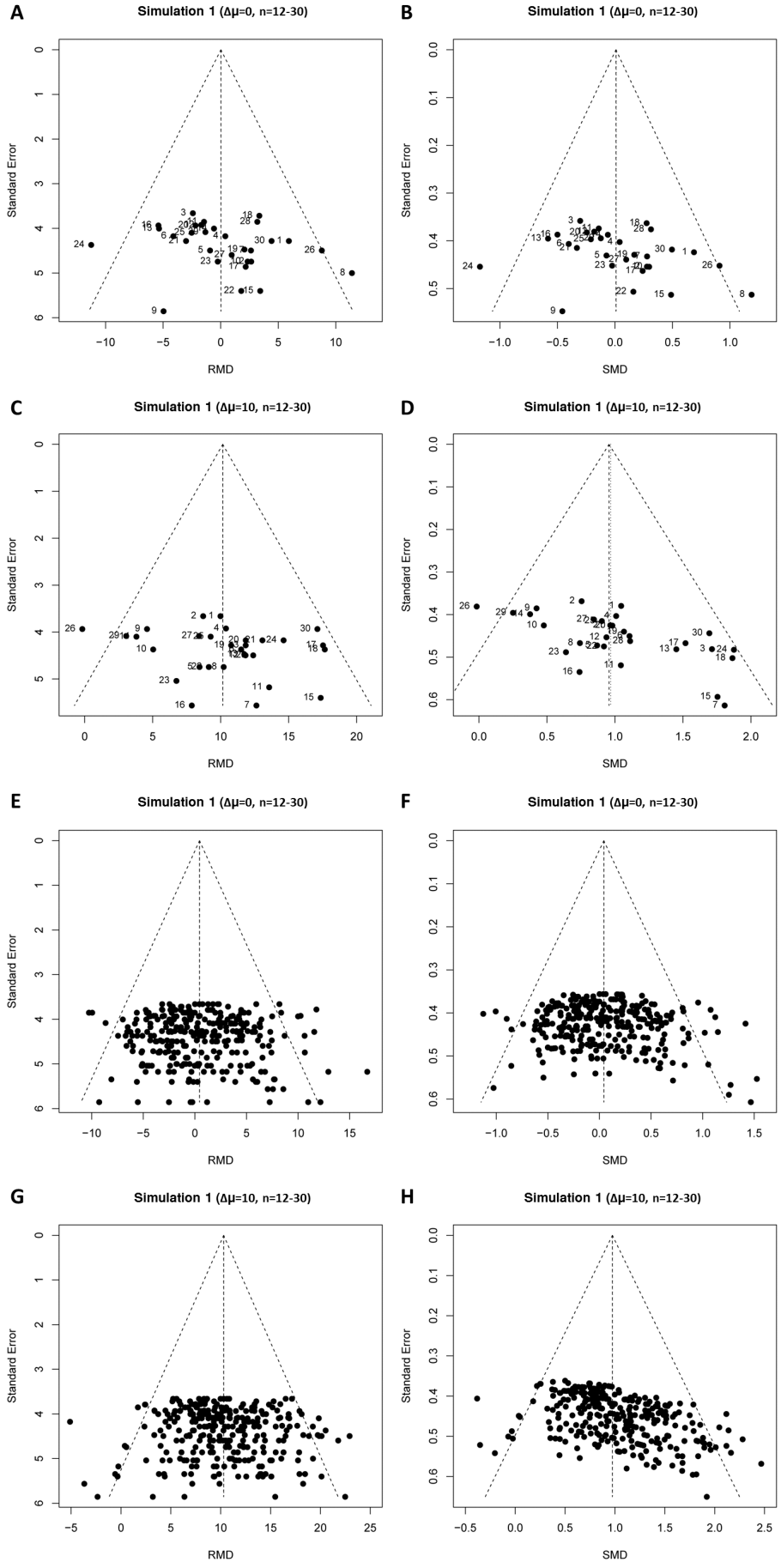


Figure 2. Representative raw mean difference (RMD; **A**, **C**, **E**, **G**) and standardized mean difference (SMD; **B**, **D**, **F**, **H**) funnel plots for simulated unbiased meta-analyses containing thirty (**A**-**D**) or 300 (**E**-**H**) studies with a small sample size (n=12-30). Simulations were performed without an intervention effect (Δμ=0; **A**-**B** and **E**-**F**), or with an intervention effect (Δμ=10; **C**-**D** and **G**-**H**). Δμ = difference in normally distributed means between control and intervention group. When repeating the simulations using Cohen's *d* SMD instead of Hedges' *g*, we found similar results (Supplemental Figure 2).

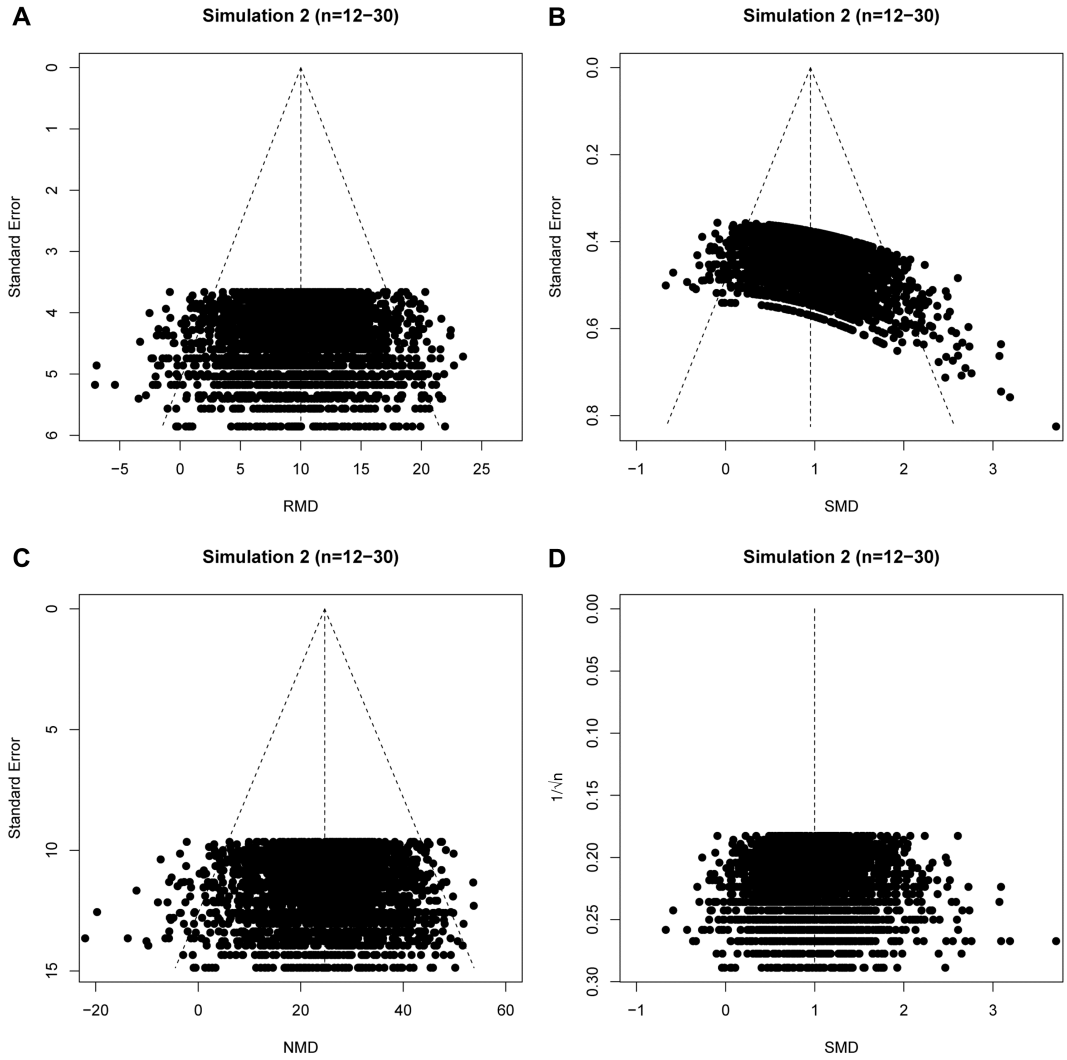


Figure 3. raw mean difference (RMD; **A**), standardized mean difference (SMD; **B**), normalized mean difference (NMD; **C**) with SE as precision estimate, and SMD funnel plots using $1/\sqrt{n}$ as precision estimate (**D**). All plots show the same simulated meta-analysis containing 3000 studies with small sample sizes (n=12-30) and an overall intervention effect ($\Delta\mu = 10$). $\Delta\mu$ = difference in normally distributed means between control and intervention group.

In our final simulation, we tested the performance of these different approaches in the presence of a simulated publication bias. In the majority of these simulations of meta-analyses of individually small studies, asymmetry was detected both visually (Supplemental Figure 3), and using Egger's regression (Supplemental Table 1). Where the size of individual studies was small, SMD-$1/\sqrt{n}$ funnel plots performed as well as the RMD-SE funnel plots, in both biased and unbiased simulations (Table 3). The NMD also behaved similar to the RMD with either an SE- or $1/\sqrt{n}$ precision estimate.

Bias?	% of simulations with Egger's p<0.05 median p-value (range)					
	RMD-SE	RMD-1/√n	SMD-SE	SMD-1/√n	NMD-SE	NMD-1/√n
No	4.6 0.52 (0.002 - 1.0)	4.5 0.52 (0.002 - 1.0)	100* $3.69*10^{-13}$ (0 - $1.8*10^{-5}$)	5.5 0.49 (0.002 - 1.0)	4.9 0.49 (0.002 - 1.0)	4.4 0.48 (0.002 - 1.0)
Yes	94.3 0.0007 ($9.9*10^{-12}$ - 0.6)	94.3 0.0007 ($9.4*10^{-12}$ - 0.6)	100* 0 (0 - $1.1*10^{-8}$)	93.9 0.0006 ($4.1*10^{-11}$ – 1.0)	91.2 0.001 ($6.8*10^{-10}$ – 1.0)	91.8 0.001 ($4.3*10^{-10}$ - 0.8)

Table 3. publication bias assessments in unbiased and biased simulations using the RMD or SMD in combination with an SE or sample size-based precision estimate (simulation 3). Simulated meta-analyses contained 300 studies (each study had 12-30 subjects) and an intervention effect was present (difference in normally distributed means between control and intervention group = 10). Publication bias was introduced by removing all studies in which the difference between the intervention and control group means was p≥0.10. N = sample size; RMD = raw mean difference; SE = standard error; SMD = standardized mean difference (Hedges' g); sims = simulations; *differs from RMD-SE p<0.004.

Study	n	Precision estimate: Standard Error – Egger's p	Standard Error – Studies filled	$1/\sqrt{n}$ – Egger's p	$1/\sqrt{n}$ – Studies filled
Egan 2016[20]	1392	$<2.2\times10^{-16}$	252	2.2×10^{-11}	0
Groenink 2014[21]	43	8.5×10^{-10}	0	0.68	0
Kleikers 2015[22]	20	3.5×10^{-4}	6	2.9×10^{-3}	0
Wever 2012[23]	62	7.8×10^{-6}	3	0.62	0
Yan 2015[24]	60	6.5×10^{-6}	0	0.19	0

Table 4. Re-analysis of published preclinical meta-analyses using SMD. N = number of studies; Egger's p = p-value for Egger's regression

Discussion

Using data from both simulated and real meta-analyses, we have shown that Egger's regression tests for funnel plot asymmetry based on plotting SMD against SE are associated with such a substantial over-estimation of asymmetry as to render this approach worthless, particularly when the size of contributing studies is small. This distortion occurs whenever a treatment effect is present, both in meta-analyses with and without publication bias. The severity of distortion and the risk of misinterpretation are influenced by the sample size of the individual studies, the number of studies in the meta-analysis, and the presence or absence of an intervention effect. Thus, the use of SMD-SE funnel plots may lead to invalid conclusions about the presence or absence of publication bias and should not be used. When using trim and fill analysis, funnel plot distortion introduces the risk of incorrectly adjusting the summary effect estimate. Previous reports of the presence of publication bias based on this approach should be re-evaluated, both for pre-clinical and clinical meta-analyses. Importantly, distortion does not occur in NMD-SE funnel plots, which formed the basis of a recent analysis showing evidence for substantial publication bias in the animal stroke literature.[25]

As the use of meta-analysis to summarize clinical and preclinical data continues to increase, continuous evaluation and development of research methods is crucial to promote high-quality meta-research.[26] Current guidance on the use of SMDs in funnel plot analysis is limited; the Cochrane Handbook for Systematic Reviews of Interventions[27] states that artefacts may occur and that firm guidance on this matter is not yet available. To our knowledge, the phenomenon of funnel plot skewing for SMDs has not yet been described in detail. It is disquieting that publication bias analyses using SMD funnel plots have been published in clinical and preclinical research areas, presumably because both the authors and the peer reviewers were unaware of the risk of spurious publication bias introduced by this methodology. Accepted papers from our group and others using SMDs for publication bias assessments have passed the peer review system, with no additional questions and or comments on this potential problem.

A similar phenomenon has been reported for the use of odds ratios in meta-analyses, which also induces artificial significant results in Egger's regression.[28] Here also an alternative test based on sample size has been proposed to circumvent this problem.[28] While it has been recommended to

use a sample size-based vertical axis for all mean difference measurements in funnel plots[10], this recommendation is not included in the Cochrane handbook or other guidelines. We agree with this recommendation for SMDs, and therefore advise not to use the SMD if it can be avoided.

Given the relative performance of the RMD, NMD and SMD approaches it is reasonable to consider whether SMD should ever be used. The RMD approach is limited because there are many instances (for example across species) where, although the same units of measurement are used, a given change may have very different biological importance. The NMD approach is preferred, but – because it expresses the effects of an intervention as a proportion of lesion size – there may be circumstances where outcome in a non-lesioned animal is not reported or cannot be inferred, and here the NMD approach is not possible.
Taken with the increased distortion seen when contributing studies are individually small, this means our findings may be especially relevant for preclinical meta-analyses. The SMD is frequently used in preclinical meta-analyses to overcome expected heterogeneity between data obtained from different animal species. Nevertheless, the SMD is also used in clinical meta-analyses and the degree of distortion cannot be readily predicted. In any case, distortion causes the threshold for determining publication bias to be artificially lowered when using SMDs and their SE, increasing the chance of false-positive results.
Of note, trim and fill analysis may not always be reliable when the number of studies in a meta-analysis is large; in half of the cases of our unbiased simulations with 300 and 3000 studies, many studies were deemed missing, even if no intervention effect was introduced. Still, the SMD simulations were always more susceptible to the addition of imputed studies if a true effect was introduced, and the effect size reduction was larger compared to RMD measurements.

Recommendations
We recommend that, where possible, investigators use RMD or NMD as an alternative to SMD in meta-analyses. Where it is necessary to use SMD, assessment for publication bias should use a sample size-based precision estimate such as $1/\sqrt{n}$. In a given analysis it may be possible to calculate an NMD effect size for some but not all studies. In these circumstances, there is a trade-off between the reduced number of included studies and an improved estimation of publication bias, and sensitivity analysis may be used to compare the meta-analysis outcome using the NMD versus the SMD. Of note, other methods to investigate publication bias in a dataset may be used in addition to funnel plots (*e.g.* fail-safe N or selection method approaches[29]), but the performance of these approaches in the context of SMD, RMD and NMD estimates of effect size is not known.
In conclusion, funnel plots based on SMDs and their SE should be interpreted with caution, as the chosen precision estimate is crucial for detection of real funnel plot asymmetry.

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Supplemental material

Supplemental equations on Hedges' *g*

Hedges' g[6] is a popular modification of Cohen's d[5] that corrects for biases due to small sample sizes. Hedges' g can be calculated by multiplying Cohen's d with the conversion factor J.

$$Hedges'g = J\ x\ Cohen's\ d$$

$$J = 1 - \frac{3}{4\ (n_{ctrl} + n_{int} - 2) - 1}$$

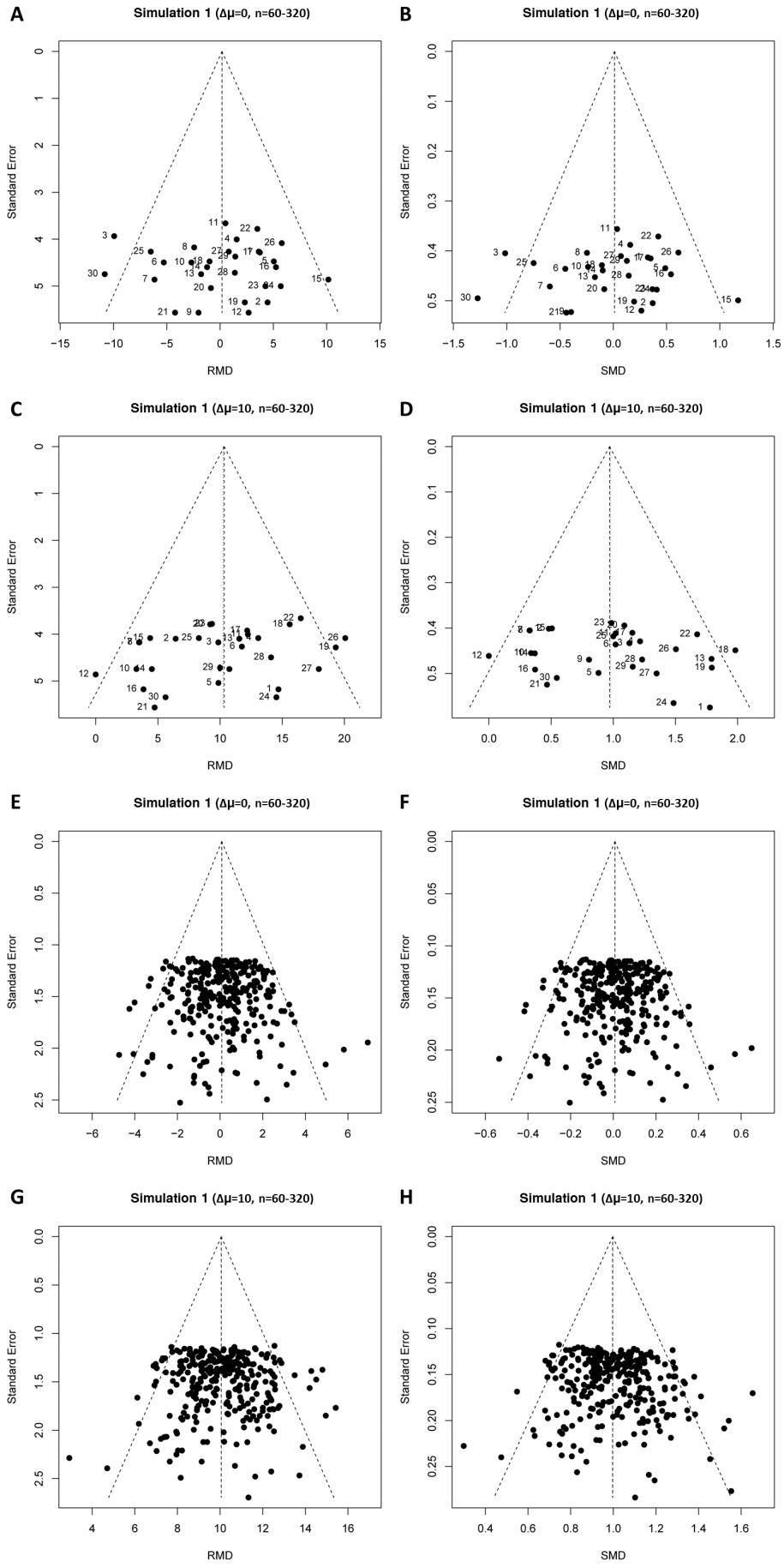
The standard error (SE) of Hedges' g can be approximated by:

$$SE_{Hedges'g} = \sqrt{J^2 * \frac{(n_{ctrl} + n_{int})}{n_{ctrl} * n_{int}} + \frac{SMD^2}{2 * (n_{ctrl} + n_{int})}}$$

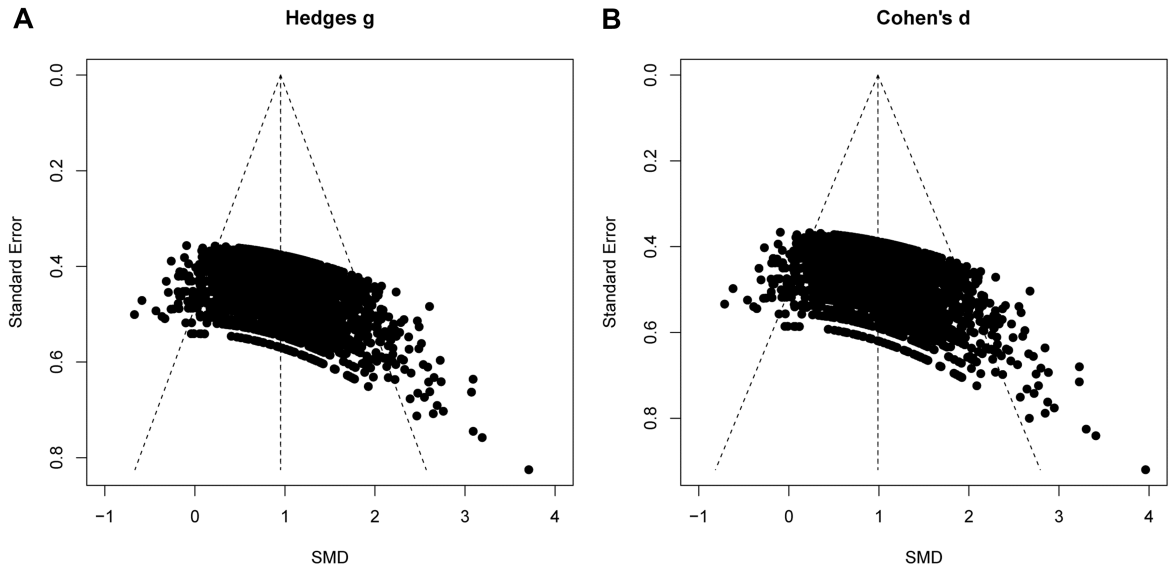
Supplemental Table 1: publication bias assessments in biased simulations using the RMD or SMD and SE (simulation 3)

Primary study n	Δμ	MA n	Egger's p<0.05 (% of sims)	Number of filled studies (mean±SD)
12-30	10	30	RMD: 83.8 SMD: 82.4	RMD: 1.2±1.9 SMD: 2.5±2.5
12-30	10	300	RMD: 92.6 SMD: 100*	RMD: 14.6±19.0 SMD: 46.6±8.6
12-30	10	3000	RMD: 100 SMD: 100	RMD: 133.6±181.4 SMD: 484.6±14.1
60-320	10	30	RMD: 8.7 SMD: 8.7	RMD: 2.4±2.7 SMD: 2.5±2.7
60-320	10	300	RMD: 4.7 SMD: 35.2*	RMD: 18.9±16.3 SMD: 28.2±16.8
60-320	10	3000	RMD: 4.6 SMD: 100*	RMD: 138.0±98.7 SMD: 333.4± 7.6

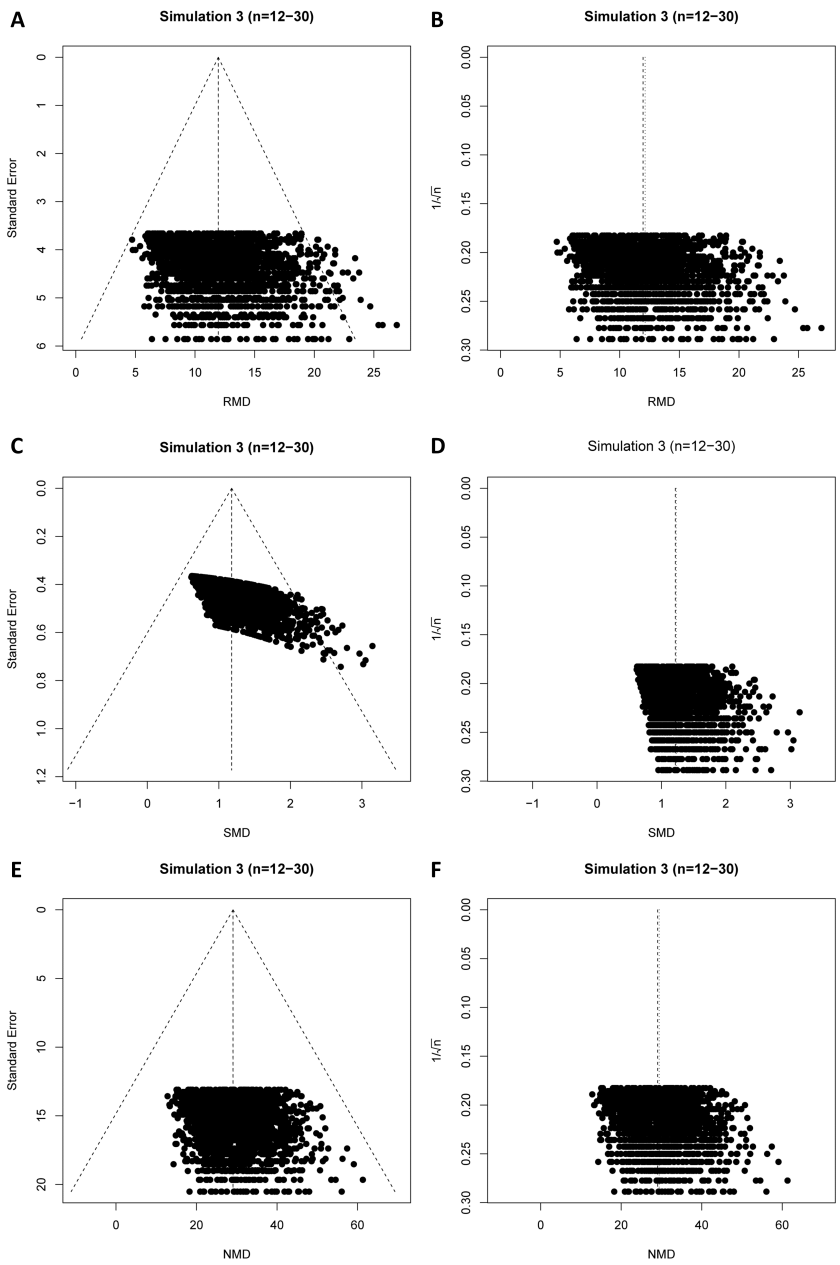
n = sample size; Δμ = difference in normal distribution means between control and intervention group; MA = meta-analysis; RMD = raw mean difference; SMD = standardized mean difference; sims = simulations; SD = standard deviation; *differs from RMD p<0.008.



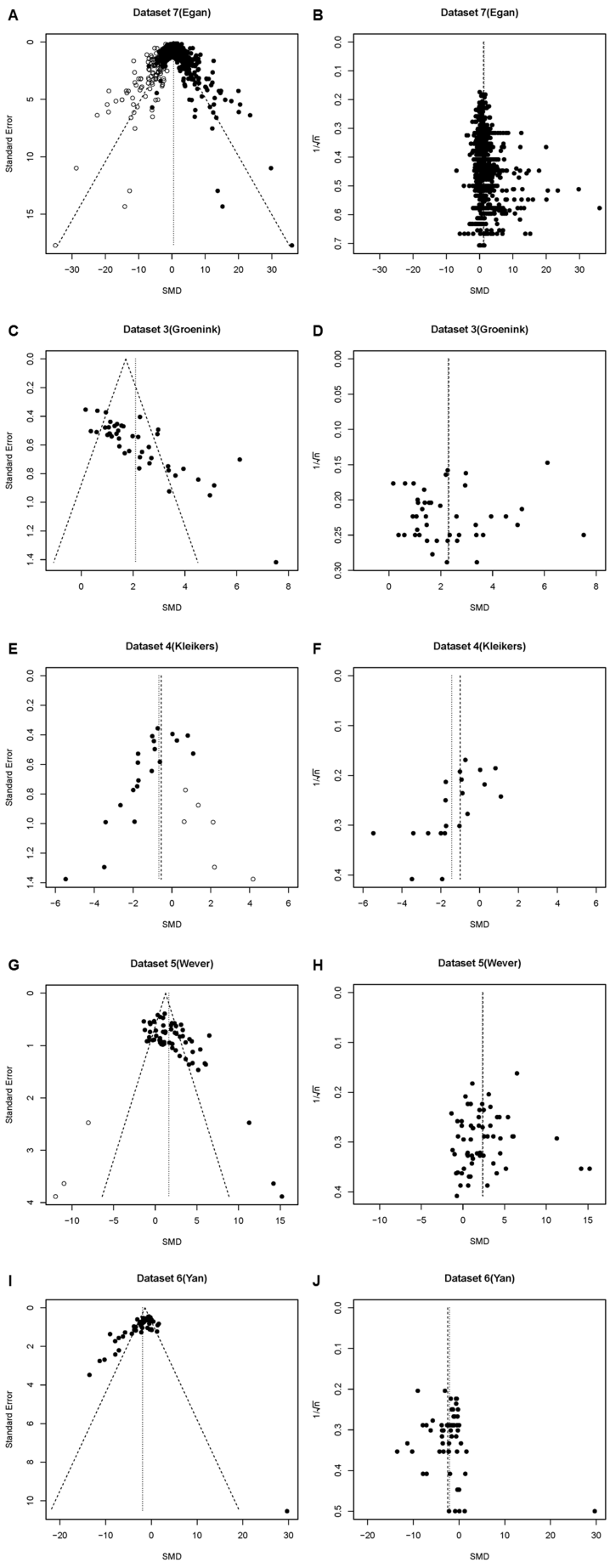
Supplemental Figure 1. Representative raw mean difference (RMD; **A**, **C**, **E**, **G**) and standardized mean difference (SMD; **B**, **D**, **F**, **H**) funnel plots for simulated unbiased meta-analyses containing thirty (**A**-**D**) or 300 (**E**-**H**) studies with a large sample size (n=60-320). Simulations were performed without an intervention effect (Δμ=0; **A**-**B** and **E**-**F**), or with an intervention effect (Δμ=10; **C**-**D** and **G**-**H**). Δμ = difference in normal distribution means between control and intervention group.



Supplemental Figure 2. Representative funnel plots of the same simulated standardized mean difference (SMD) meta-analysis of 3000 studies using Cohen's d (A) *versus* Hedges g (B). No differences in outcome were observed.



Supplemental Figure 3. Representative funnel plots of simulated biased meta-analyses using a raw mean difference (RMD; **A-B**), a standardized mean difference (SMD; **C-D**), or a normalised mean difference (NMD; **E-F**) effect measure. The present example contains 3000 studies with a small study sample size (n=12-30) and an intervention effect present (difference in normal distribution means between control and intervention group = 10). Publication bias was introduced by removing all studies in which the difference between the intervention and control group means was p≥0.10. Precision estimates are standard error (**A, C, E**) or sample size-based (**B, D, F**), where n = total primary study sample size.



Supplemental Figure 4. Funnel plots of empirical meta-analyses plotted as standardized mean difference (SMD) *versus* standard error, as in the original publications (left hand panels), and as SMD *versus* $1/\sqrt{n}$ after re-analysis. n = total primary study sample size; filled circles = observed data points; open circles = missing data points as suggested by trim and fill analysis.

11

Meta-analysis of control groups in cardiovascular cell therapy trials suggests reparative responses through sham-procedures

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In preparation

ABSTRACT

Background

Cell therapy has so far failed to be approved for standard clinical care after ischemic heart disease. Its efficacy has been tested in many placebo-controlled trials, using sham interventions for bone marrow (BM)-punctures and placebo administration. Although sham interventions are considered the golden standard to account for placebo-effects, we hypothesized that sham interventions in cell therapy might also have an additional biological effect. Here we use the heterogeneity in clinical trial design to compare functional data of subjects from control arms and compare invasive sham interventions to their non-placebo treated counterparts.

Methods

Pubmed and Embase were searched for all relevant clinical trials for ischemic heart disease, using autologous cellular BM therapeutics. Effect sizes for ejection fraction and infarct size at baseline and follow-up were extracted. Variables of interest were (1) disease type (acute vs chronic) (2) sham placebo administration, (3) post-conditioning during administration and (4) the use of sham BM-puncture. We performed meta-analysis and meta-regression for our variables of interest. To biologically support our findings, we also directly investigated the direct mobilization of progenitor cells after BM-puncture in fresh patient material.

Results

We retrieved 4897 articles, from which 52 made the final selection. Type of ischemic disease seems to significantly influence outcomes over time in both control and therapy groups. Studies using placebo administration ($p=0.03$) and post-conditioning ($p=0.01$) showed a significant positive effect in control subjects on infarct size and no effect on ejection fraction gains ($p=0.71$ and $p=0.08$). There was no difference observed on cardiac function ($p=0.93$) and infarct size ($p=0.96$) when BM-puncture was performed. This was confirmed in fresh blood samples after BM-puncture in a small cohort study, showing no increase in progenitor cell mobilization *in vivo*.

There were no significant differences in the therapy groups for any of the variables.

Conclusion

Our data suggests an added effect of placebo administration and post-conditioning in control groups of cell therapy trials. This phenomenon might have a biological substrate, which adds to any drug administered and potential placebo effects. There seems to be no acute mobilization of progenitor cells after BM-puncture, which also did not show any improvement when performed as a sham procedure in cell therapy trials.

Abbreviations

IHD	ischemic heart disease
MI	myocardial infarction
BM	bone marrow
CHD	chronic heart disease
LVEF	left ventricular ejection fraction
IS	Infarct Size

Introduction

Cell therapy has been clinically introduced over a decade ago as a therapy for ischemic heart disease (IHD). However, there is still debate on its efficacy and added value on top of standard clinical care, despite many randomized controlled trials and at least as many meta-analyses.[1-4] There is substantial heterogeneity in treatment efficacy, both within- and between trials. For autologous bone marrow (BM)-derived cell products, this variability comes from both disease related factors, such as infarct size, but also from factors related to the autologous cell product, indicating a complex interplay between BM and the infarcted heart.[5,6] Recent studies have shown that BM composition changes immediately after myocardial infarction (MI)[7] and that BM characteristics determine functional outcome after MI and in chronic IHD.[7,8]

The reciprocal relationship between the infarcted heart and the BM implies some form of biological connection, which may also be of importance to the mechanism behind cell therapy. It is conceivable that manipulation of the BM-compartment in any form may affect outcome after IHD. Coronary artery bypass grafting surgery, including a sternotomy, results in an increase of circulating progenitor cells.[9] Traumatic bone injury has also been shown to mobilize various types of progenitor cells into circulation[10,11], and there is a well-established link between fractures and previous MI.[12] It is therefore conceivable that the BM-aspiration itself in autologous BM-mononuclear cell therapy modifies functional outcome. The process of post-conditioning is also known to positively affect outcomes after IHD.[13,14] These conditioning approaches are known to positively affect the injured myocardium, potentially inducing an extra endogenous repair response.[15] Interestingly, during intracoronary administration of cell therapy (or a placebo treatment) many groups made use of stop-flow techniques[16], therefore hypothetically also inducing some form of post-conditioning during drug infusion.

A recent meta-analysis of stem cell trials for ischemic heart disease stratified for the use of sham controls in any trial, showing reduced efficacy of cell therapy in studies using sham interventions for BM-aspiration or placebo administration in controls compared to studies using no sham procedures.[17] As this meta-analysis analyzed treatment and control arms in a paired fashion, it does not show whether the effect between control arms comes from an increase or decrease in therapy groups or control groups in the individual studies. For this study, we hypothesized that sham-control procedures have an added biological and 'therapeutic' effect on primary outcomes in cardiac cell therapy trials on top of a regular placebo effect. On top of a placebo effect, potential biological mechanisms could be altered reactions and homeostasis in the BM upon puncture or effects of(sham-)administration procedures, for example through post-conditioning.[18,19] Here we employ meta-analysis to investigate potential biological effects of sham interventions in the control arms of bone-marrow cell therapy trials, comparing these groups to the incorporated therapy groups. We use the progression of left ventricular ejection fraction (LVEF) and infarct size (IS) over time after intervention as primary outcome measures. Groups are stratified on the presence of a BM-puncture, the use of placebo administration and post-conditioning in the control arm of the trial. In a small cohort study, we investigate the immediate effect of BM aspiration on circulating progenitor cell levels in fresh samples.

Methods

Systematic search for BM studies treating cardiac ischemic disease

We performed a literature search of PubMed and Embase, that was adapted from a previous publication.[20] The complete search strategy, including synonyms, can be found in the Supplementary Methods. In short, papers were screened by two investigators (PPZ, MK) in both title-abstract and full-text phase. A third investigator (HG) was consulted in case of no consensus. Included papers were clinical studies including a control group, on cardiac ischemic disease, using any form of autologous BM-derived cellular therapeutic. Measurements on ejection fraction and infarct size were extracted, for which papers needed to have at least an outcome measure on a baseline and follow-up time point. For our primary analysis, studies reporting a follow-up of 2-6 months were included. If multiple measurements were done in this time period, the latest measurement was used. If multiple publications of the same trial existed that reported on the same time point, only the publication reporting most individuals measured was used. If different therapy groups were studied in one study, both therapy groups were incorporated and control subjects were divided equally over these groups. Importantly, this results in more comparisons than there are studies in our analyses. From baseline and follow-up measurements, a raw mean difference was generated for both control and therapy groups. Variables for our meta-regression were extracted per study and included (1) disease type, (2) use of a sham BM-puncture, (3) sham placebo infusion/injection and (4) the use of post-conditioning either in the therapy group or in both groups. For disease type we distinguished acute MI and chronic ischemic disease (CHD), where CHD was considered all interventions more than 14 days after MI, chronic ischemia and coronary artery disease.

Statistical analysis

We performed a random-effects meta-analysis, as there is considerable expected heterogeneity within included studies. Meta-analysis was performed using the DerSimonian Laird approach. Specific heterogeneity could occur between disease types (MI and CHD), which was first tested. Meta-regression for other variables of interest was performed univariably and a sensitivity analysis was performed correcting for disease type (acute or chronic ischemic disease). The analysis was performed using R version 3.1.2 with the addition of the meta package.[21,22]

BM study population and protocol

Samples were collected for a study which examined BM-derived progenitor cell function in chronic kidney disease. BM-aspirate, iliac crest biopsies and peripheral blood were obtained from 38 patients (21 kidney donors and 17 kidney recipients) participating in the living donor kidney transplantation program at UMC Utrecht. Peripheral blood samples taken within 10 minutes of BM-aspiration were available for 19 patients (9 kidney donors and 10 kidney recipients). BM biopsy took place after induction of anesthesia for the kidney donation / transplantation procedure. 20mL peripheral blood (10 mL before and 10mL after the biopsy) and 20mL BM were collected in EDTA and heparin coated vacuum tubes respectively and stored at room temperature until analysis. The iliac crest biopsy was stored in a 4% formalin solution.

Flow cytometry

50 µl of blood or BM was stained with an antibody cocktail to enumerate circulating progenitors. Staining was performed for 30 minutes at 4° Celsius in the dark in the presence of blocking reagent. Erythrocytes were subsequently lysed and dead cells were stained. Each specimen was stained in duplicate and 30.000 events (PB) or 10.000 events (BM) were acquired on a BD FACSCantoII flow cytometer. Analysis was performed using FlowJo software (Treestar, version 10.0.6). Cell numbers are expressed as cells per µl for blood. A complete blood count of blood and BM was made separately on a hematology analyzer (Abbott Cell-dyn 1800).

Results

The search was performed on February 8th 2016. 4897 papers were extracted (Supplementary Figure 1). After duplicate removal and title-abstract screening, 373 papers were eligible for full text screening. After full-text screening, we included 52 papers for our primary analyses. Two studies were partially excluded for specific analyses (see Supplemental Methods). Meta-analysis of all included control groups revealed an increase in cardiac function over time (n=59 comparisons) of 2.81% (95% CI 1.71 – 3.04, $p=2.4*10^{-12}$) (Supplementary Figure 2), while meta-analysis for infarct size (n=34 comparisons) revealed a general decrease in infarct size over time of -4.31 (95%CI -5.84 - -2.77, $p=3.9*10^{-8}$) (Supplementary Figure 3).

<u>Meta-regression suggests differences in acute and chronic disease in control and therapy groups</u>

We first performed meta-regression for disease type, as we expected substantial heterogeneity when comparing MI and CHD studies. Meta-regression confirmed a larger increase in cardiac function after baseline measurements in MI compared to CHD in both control groups and cell-treated groups (Figure 1A-B, $p<0.0001$ and $p=0.03$ respectively). In line with this, infarct size reduction was significantly larger over time for MI compared to CHD in both control and therapy groups (Figure 1C-D, $p= 0.004$ and $p=0.02$ respectively).

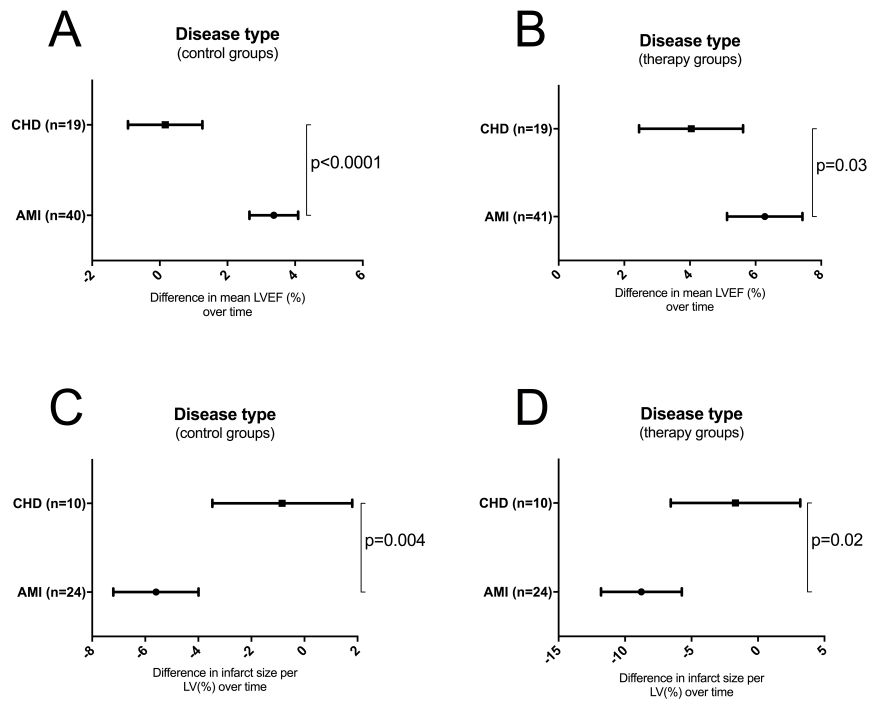

Figure 1. Meta-regression for disease type reveals comparable functional gains in therapy and control groups, with differences between disease types (A) Therapy groups. (B) Control groups.

<u>Meta-regression reveals changes in controls after placebo administration and post-conditioning</u>

We performed meta-regression for all variables of interest. Our analyses revealed that BM-aspiration had no effect on cardiac function in control groups (Figure 2A, p=0.82), which did not change after correction for disease type (p=0.65). There was also no effect of BM-aspiration on infarct size reduction in control groups (Figure 3A, p=0.93 and p=0.74 in sensitivity analysis).

Post-conditioning showed a non-significant increase in cardiac function in control groups compared to non-post-conditioned control subjects (Figure 2B, p=0.08), which after disease correction was far from significant (p=0.29). For infarct size, use of post-conditioning did show a significant change of -7.5% compared to -3.3% when not applied (Figure 3B, p=0.009 and p=0.02 in sensitivity analysis).

Administration of a placebo solution did not change ejection fraction in control subjects significantly (Figure 2C, p=0.71), which remained similar after our sensitivity analysis for disease type (p=0.92). Placebo administration did change infarct size significantly, with a decrease of -6.8% when administered, compared to -3.4% when not (Figure 3C, p=0.03 and p=0.03 in sensitivity analysis).

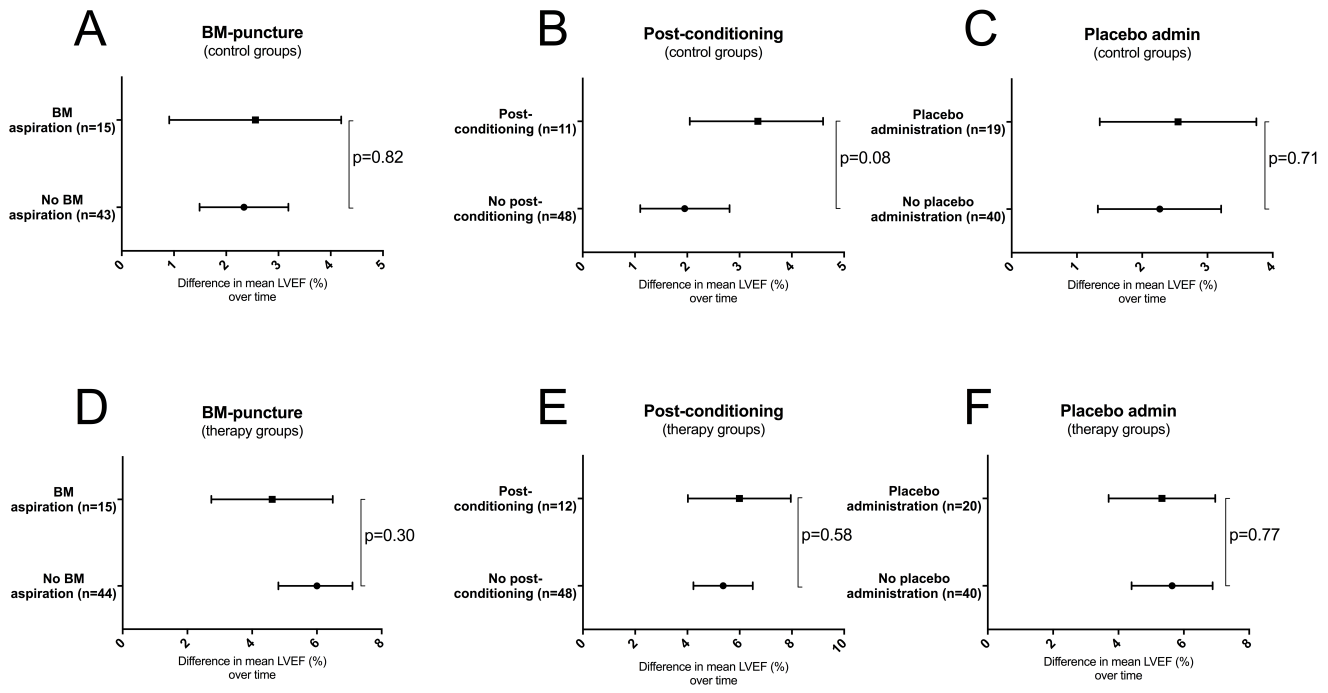

Figure 2. Meta-regression analysis on cardiac function improvement for all variables of interest on control groups and therapy groups. (A) bone marrow aspiration (control groups), (B) post-conditioning (control groups), (C) placebo administration (control groups), (D) bone marrow aspiration (therapy groups), (E) post-conditioning (therapy groups), (F) placebo administration (therapy groups).

In all therapeutic groups, we did the same analyses. Grouping was still based on the variable of interest in the linked control groups; meaning the procedure was or was not performed in the accompanying control group. As expected, there was no difference in cardiac function for therapeutic groups, when control groups received a BM-aspiration, placebo administration or post-conditioning (Figure 2D-F, p=0.30, p=0.58 and p=0.77, respectively). Sensitivity analyses with correction for disease type did not change these results (p=0.49, p=0.90 and p=0.68, respectively). With regards to infarct size, there were also no significant differences in therapy groups when control groups underwent BM-aspiration, placebo administration or post-conditioning (Figure 3D-F, p=0.13, p=0.91 and p=0.91, respectively with no changes in the sensitivity analyses worth mentioning).

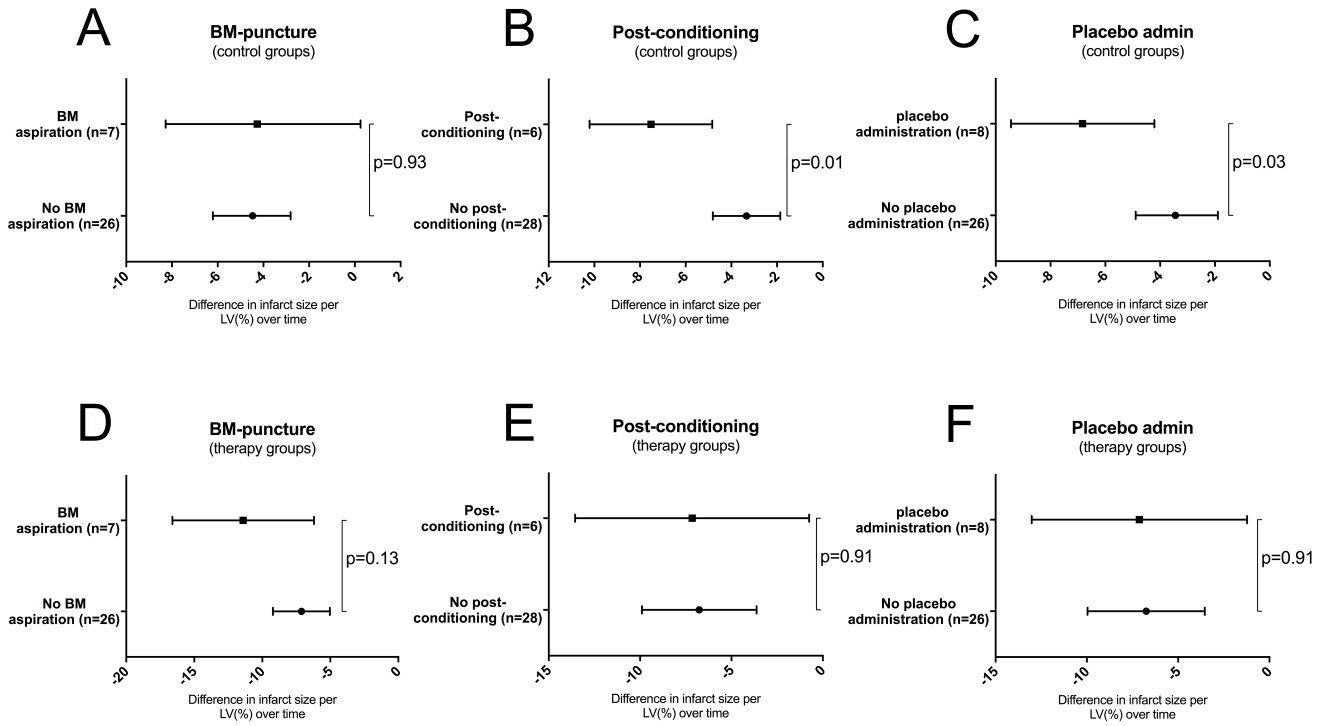

Figure 3. Meta-regression analysis on infarct size reduction for all variables of interest on control groups and therapy groups. (A) bone marrow aspiration (control groups), (B) post-conditioning (control groups), (C) placebo administration (control groups), (D) bone marrow aspiration (therapy groups), (E) post-conditioning (therapy groups), (F) placebo administration (therapy groups).

<u>Progenitor cell mobilization does not seem affected by BM-aspiration</u>

In chronic kidney disease patients undergoing BM-aspiration, we measured $CD34^+$ cells before and directly after puncture (within 10 minutes). Upon FACS-sorting there were no difference observed in $CD34^+$ cell mobilization (Figure 4, p=0.39).

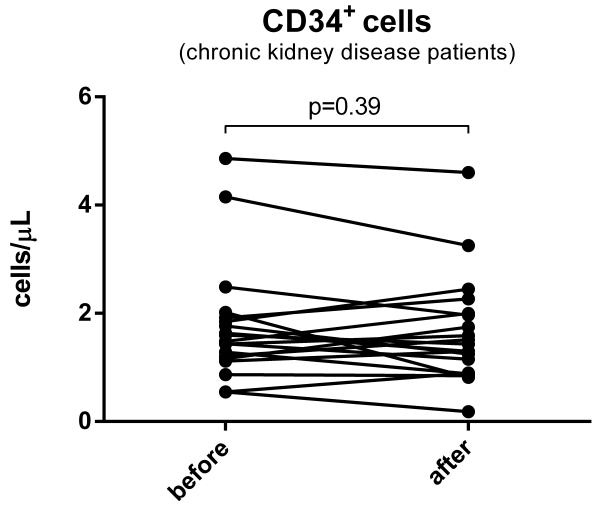

Figure 4. No increase in $CD34^+$ cells after BM-aspiration in chronic kidney disease patients

Discussion

In the present study, we used the heterogeneity in clinical trial design to investigate the potential

biological effects of different sham-interventions in stem/progenitor cell trials for IHD. After correction of differences in the natural disease history between MI and CHD, we found indications for increased recovery in patients that underwent placebo administration and post-conditioning. No indications for an effect of BM-aspiration on functional outcome were observed. In an additional small cohort study, we showed that BM-aspiration does not induce appreciable mechanical mobilization of progenitor cells. Patients in control arms improve significantly after an acute MI and trial participation, which is in line with observatory data on infarct size decrease and cardiac function improvements after MI.[23-26] Cell therapy studies in MI without control arms reporting gains of function should exceed these numbers, as mean marginal gains are to be expected in many MI patients.

Our results do not support the existence of a biological effect of BM-aspiration procedures. This is also not supported by our lab results, showing no direct mobilization of progenitor cells after BM-puncture. Interestingly, although distant acute damage like MI[27,28] and chronic disease like diabetes[29] or obesity[30,31] cause direct changes in circulating progenitor distributions, our data cannot confirm this for direct damage through puncture to the BM after MI. This might also be due to the reaction to ischemia itself (at least in the case of acute MI studies), with little room for the BM to react even more.

Our analyses suggest a role for placebo administration and post-conditioning in cell therapy trials, potentially giving control groups a reparative boost, affecting primary efficacy measurements. These groups are partially overlapping in our dataset, as intracoronary placebo administrations usually include a stop-flow technique, while intramuscular injections do not. As timing matters for post-conditioning[19,32,33], a decreasing effect upon later administration in both control and therapy groups cannot be excluded, potentially affecting this phenomenon. However, recent evidence suggests that hypoxic periods at later time points can also induce regenerative responses in the injured heart.[34] Our data might suggest the same, as many trials administered their sham procedure many days after the ischemic event, potentially aiding in a later reparative responses that decrease a myocardial scar over time. Furthermore, it has been postulated that post-conditioning might benefit cell therapy after IHD in general, showing increased engraftment and improved efficacy on top of cell therapy alone.[35] As some of the included studies used post-conditioning in only the therapy groups, these assumptions are beyond our presented analyses.

Sham-procedures are the most appropriate way to control for any effect that is not directly attributed to the intervention under study, either through biological or placebo-mechanisms. In the case of cell therapy, both BM punctures and sham catheterizations are invasive procedures with serious potential side-effects, which raise both ethical and procedural difficulties.[20] In light of currently recruiting trials, like the BAMI-trial (www.bami-fp7.eu), which compares BM-derived cell therapy to standard clinical care, it is comforting that there does not seem to be a direct effect of BM-aspiration on standard primary outcome measures. However, a potential biological effect of the administration procedure itself on infarct size might be present, based on the hints provided by our analyses. It seems an effect seen in those specific trials can be attributed to the complete therapeutic intervention under study, which involves BM-derived cell therapy itself alongside the administration procedure.

Acknowledgements

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Supplemental Methods

Search

I – Search strategy PubMed

(((((((heart[Title/Abstract] OR cardia*[Title/Abstract] OR myocardia*[Title/Abstract] OR coronary*[Title/Abstract] OR cardiomyopath*[Title/Abstract]))
AND
((failure[Title/Abstract] OR decompensation[Title/Abstract] OR infarction[Title/Abstract] OR ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract] OR disease[Title/Abstract] OR dysfunction[Title/Abstract] OR disfunction[Title/Abstract] OR angina[Title/Abstract])))
AND
((stem*[Title/Abstract] OR progenitor*[Title/Abstract] OR (bone[Title/Abstract]
AND marrow*[Title/Abstract]) OR precursor*[Title/Abstract])))) AND
cell*[Title/Abstract]))
AND
((transcoronar*[Title/Abstract] OR intracoronar*[Title/Abstract] OR transendocardial*[Title/Abstract] OR intramyocardial*[Title/Abstract] OR intravenous*[Title/Abstract] OR transvenous[Title/Abstract]))

II – Search strategy Embase

#1: heart:ti,ab OR cardia*:ti,ab OR myocardia*:ti,ab OR coronary*:ti,ab OR cardiomyopath*:ti,ab
#2: failure:ti,ab OR decompensation:ti,ab OR infarction:ti,ab OR ischemi*:ti,ab OR ischaemi*:ti,ab OR
disease:ti,ab OR dysfunction:ti,ab OR angina:ti,ab
#3: stem*:ti,ab OR progenitor*:ti,ab OR (bone:ti,ab AND marrow*:ti,ab) OR cardia*:ti,ab OR precursor*:ti,ab
#4: cell*:ti,ab
#5: transcoronar*:ti,ab OR intracoronar*:ti,ab OR transendocardial*:ti,ab OR intramyocardial*:ti,ab OR
intravenous*:ti,ab OR transvenous:ti,ab

Inclusion and exclusion criteria

For inclusion of studies, we used the following criteria:
We included all human trials conducted in adults >18 years, studying autologous BM-derived product transfer in IHD. Co-interventions, like coronary artery bypass grafting, percutaneous interventions were permitted. Any route of administration was considered. Primary outcomes were ejection fraction and infarct size over time, measured as a baseline measurement and follow-up in 2-6 months. Any modality for cardiac function (echocardiography, LV angiography, MRI, SPECT, etc) was deemed suitable. The study needed to have a control group.

We excluded studies that:

- Were not in English
- Did not have an accessible full-text paper

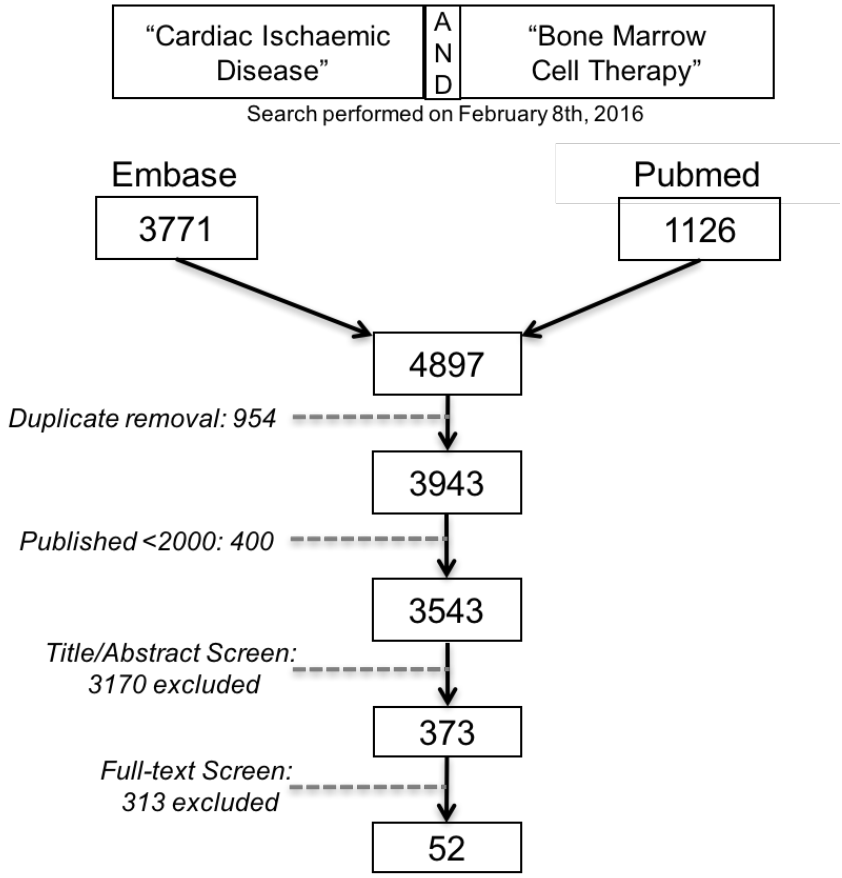
- Had a historical control group
- Were part of an already included study, with fewer included subjects for the same timepoint
- Studies from which no data could be extracted or recalculated, either from text or from figures

Special post-hoc exclusions after generation of the search protocol
One study was partially excluded as it was not clear if BM-aspiration was performed in the control group, so we left the study out for that specific meta-regression.[36] One study showed a clear discrepancy on baseline cardiac function in control groups, so was left out in the analyses investigating cardiac function in the control groups.[37]

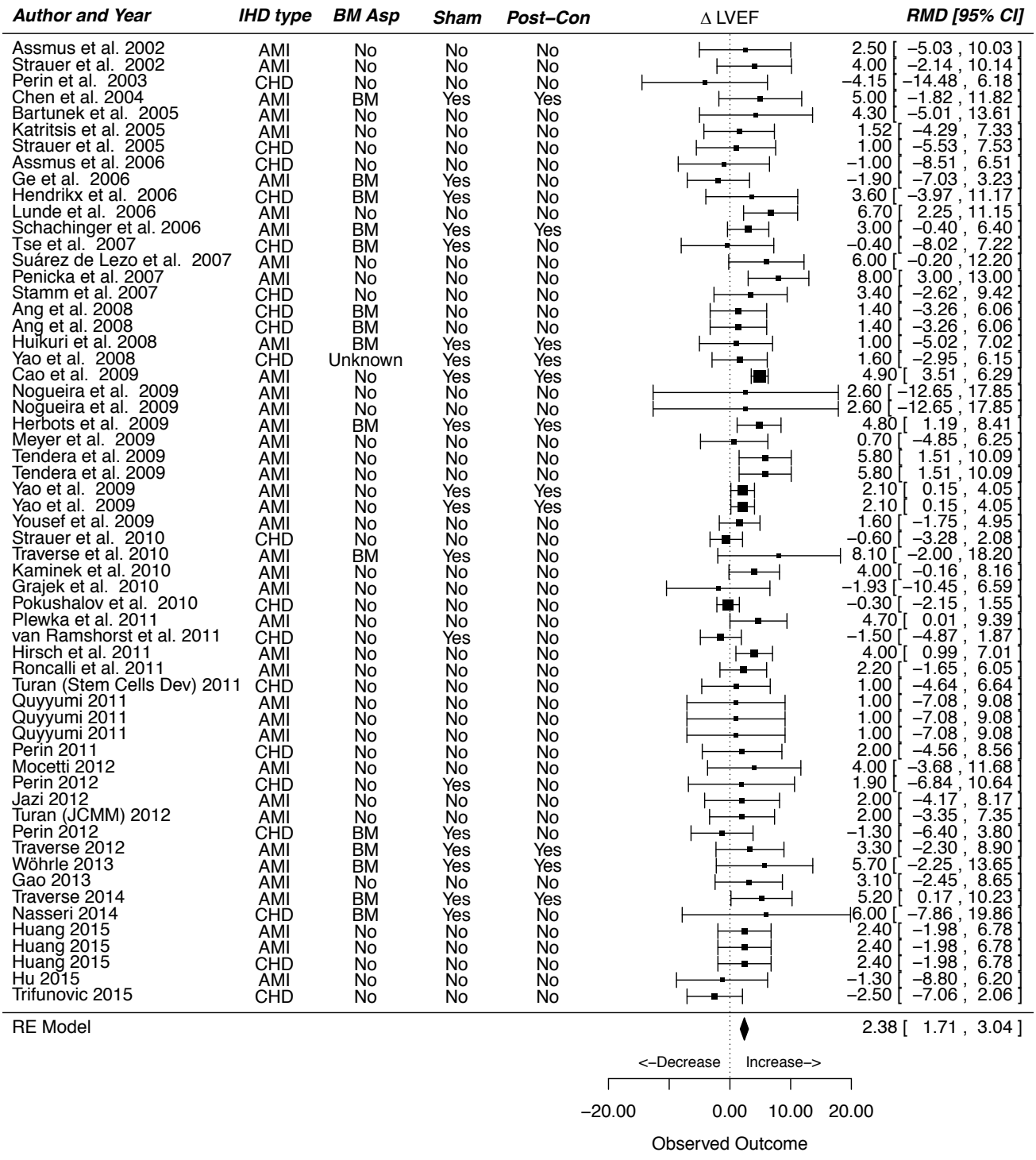
B*M study population and protocol*
Samples were collected for a study which examined BM-derived progenitor cell function in chronic kidney disease. The study complied with the Declaration of Helsinki and was approved by the local institutional review board (METC number 12-127). Written and verbal informed consent was provided by all subjects. Exclusion criteria for chronic kidney disease patients (kidney recipients) were stem cell transplantation in the past and consisted of the exclusion criteria for renal transplantation for chronic kidney disease (active infection: hepatitis B and C, tuberculosis, HIV; life expectancy <2 years; malignancy not curatively treated). Exclusion criteria for healthy controls (kidney donors) were stem cell transplantation in the past and present kidney disease. BM-aspirate, iliac crest biopsies and peripheral blood were obtained from 38 patients (21 kidney donors and 17 kidney recipients) participating in the living donor kidney transplantation program at UMC Utrecht. Peripheral blood samples taken within 10 minutes of BM-aspiration were available for 19 patients (9 kidney donors and 10 kidney recipients). BM biopsy took place after induction of anesthesia for the kidney donation / transplantation procedure. BM biopsy was conducted in accordance with the standard operating procedures at the UMC Utrecht hematology department, using a T-lok BM biopsy needle. 20mL peripheral blood (10 mL before and 10mL after the biopsy) and 20mL BM were collected in EDTA and heparin coated vacuum tubes respectively and stored at room temperature until analysis. The iliac crest biopsy was stored in a 4% formalin solution.

Flow cytometry
Circulating and BM-resident cell populations were enumerated using a 'lyse-no-wash' protocol, using BD TRUCOUNT tubes (BD Biosciences; NJ, USA) in order to allow volumetric analysis. 50 µl of blood or BM were stained with an antibody cocktail consisting of r: FITC-conjugated mouse anti-human CD34 (BD Pharmingen), phycoerythrin (PE) conjugated mouse IgG anti-hVEGF/KDR (R&D Systems; Abingdon, UK), Allophycocyanin-conjugated anti-CD133 (MACS Miltenyi Biotec; Bergisch Gladbach, Germany) and PE-Cy7 conjugated mouse anti-human CD45 (BD Pharmingen; NJ, USA) to enumerate circulating progenitors. Staining was performed for 30 minutes at 4° Celsius in the dark in the presence of FcR blocking reagent (Miltenyi). Erythrocytes were subsequently lysed using an ammonium chloride based lysis buffer. Sytox Blue® (Invitrogen Molecular Probes; Bleiswijk, the Netherlands) was added in order to stain dead cells.
Each specimen was stained in duplicate and 30.000 events (PB) or 10.000 events (BM) were acquired on a BD FACSCantoII flow cytometer. Gates were set using fluorescence -minus-one

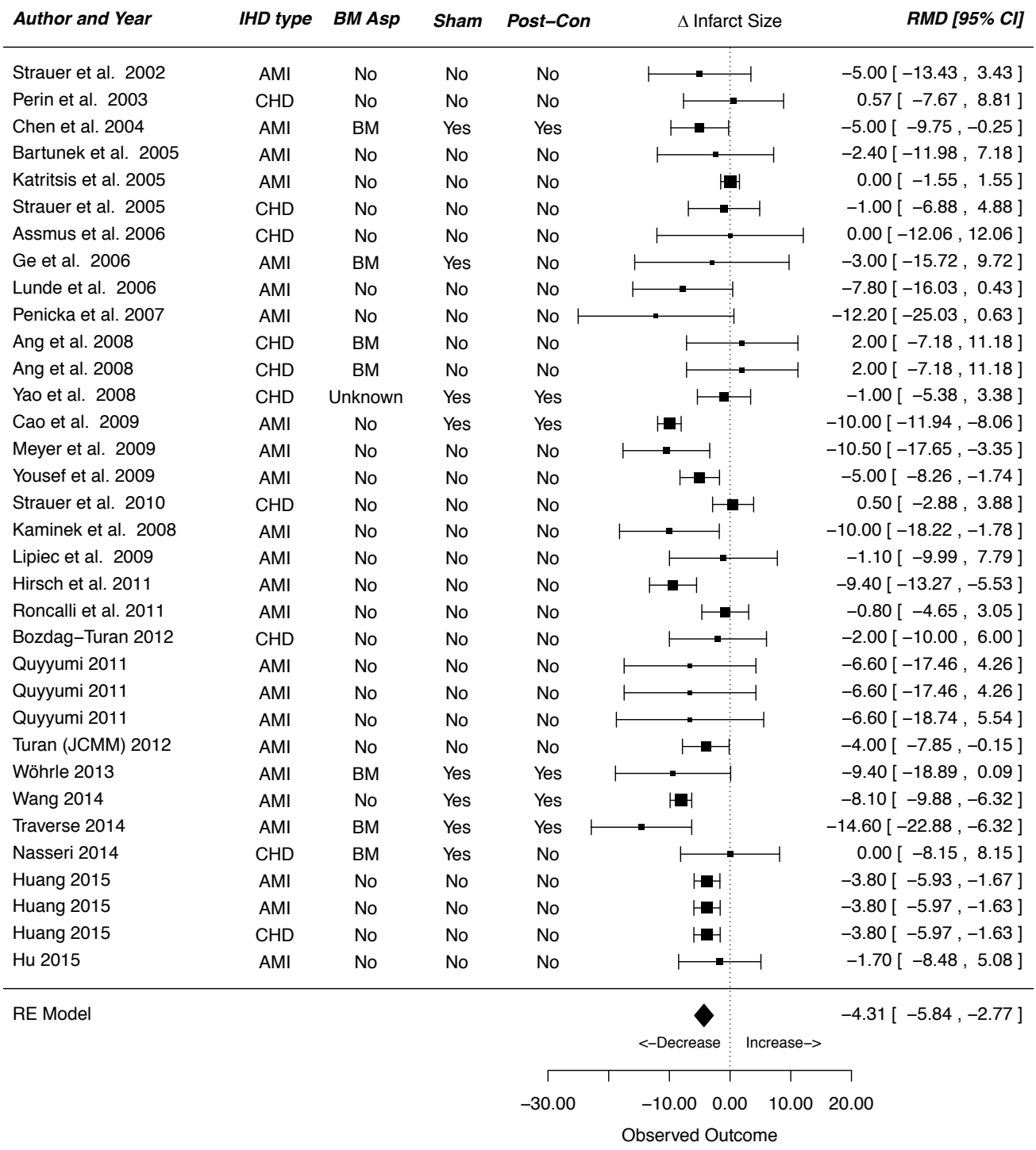
controls. Analysis was performed using FlowJo software (Treestar, version 10.0.6). Cell numbers are expressed as cells per µl for blood. A complete blood count of blood and BM was made separately on a hematology analyzer (Abbott Cell-dyn 1800).



Supplementary Figure 1. Flowchart of the conducted search and subsequent article screening.



Supplementary Figure 2. Forest plot for all control groups and ejection fraction measurements. Ejection fraction seems significantly increased over time in control subjects of cardiac cell therapy trials.



Supplementary Figure 3. Forest plot for all control groups and infarct size measurements. Infarct size seems significantly decreased over time in control subjects of cardiac cell therapy trials.

12

Responder definition in clinical stem cell trials in Cardiology; will the real responder please stand up?

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Non-standard Abbreviations and Acronyms

BMMNCs	Bone Marrow Mononuclear Cells
MI	Myocardial Infarction
EF	Ejection fraction
ΔEF	Difference in ejection fraction between baseline and 4 months follow-up
EDV	End diastolic volume
ESV	End systolic volume

Introduction

Defining responders to cell treatment based on functional measurements in cardiac stem cell trials has been troublesome, and it may be considered as the Holy Grail. The functional recovery after myocardial infarction (MI) can range from only mild impairments and recovery to progression into heart failure at the next clinical visit regardless of the therapy given. In a clinical trial with adequate randomization, this will not pose an issue on the overall outcome of the trial. However, subgroup analyses become difficult, as the whimsical course of the disease influences the end-result on top of the effect of the cell treatment. In other words, even patients who have suffered significant loss of functional cardiac capacity may still have benefited from cell therapy compared to the potential 'reference point' of the same patient in the placebo group. Among other reasons, this can make subgroup analyses hard to interpret and potentially less informative. Proper subgroup analyses are ideally addressing true response, based on (pre)clinical hints and are prospectively declared. Furthermore, the power needed to show specific responding groups might be beyond the number of participants included in hitherto conducted cell therapy trials.

Meta-analyses including all randomized controlled studies have consistently shown significant positive effects of treatment with bone marrow mononuclear cells (BMMNCs) after MI. Stratified subgroup meta-analyses hint towards different effects with increasing age and/or specific functional parameters.[1] Researchers have questioned the availability and quality of autologous cells harvested from patients with multiple risk factors.[2] The negative effects of endogenous risk factors on bone marrow- and circulating progenitor cells have been confirmed with regards to age, smoking, heart failure, diabetes and general risk factor profiles.[2] To date, it is not known if the negative effect of clinical risk factors on BMMNC function and the recipient heart is also reflected in the outcomes of clinical studies. Furthermore, the invasiveness and cost of BMMNC therapy call for better prediction of treatment response after MI. In the present analysis, we demonstrate a method based on multivariable statistical interactions, which is able to identify potential treatment responders, while simultaneously correcting for relevant factors that affect general disease outcome. With the identification of components that positively influence the (probability of a) functional gain after cell therapy, it might be possible to predict who the real responders are.

Methods and results

As a proof-of-concept, we used the data from the REPAIR-AMI trial; a multicenter randomized controlled trial, conducted from April 2004 till October 2005.[3] In the REPAIR-AMI trial difference in ejection fraction after 4 months compared to baseline (ΔEF) was used as the initial primary outcome. Patient characteristics, baseline imaging and cell characteristics were recorded. 204 patients were randomized, of which 186 had complete data after 4 months for functional outcome and characteristics.

This post-hoc analysis was not prospectively declared, but initiated and executed by independent researchers not affiliated with the primary study team. On the basis of an *a priori* power analysis, we defined 18 variables as possible predictors, based on previous literature and clinical expertise, having one variable per 10 outcome measures as is generally accepted.[4] We applied linear

regression analyses with ΔEF as outcome and the statistical interaction of cell therapy with the single possible predictors as variable of interest. These interaction terms resemble the difference between the cell treated and placebo group, regardless of the effect of the variable on the functional outcome itself. A significant interaction therefore identifies predictors in which the effect of cell therapy compared to the placebo is different within groups.

Next, we performed multivariable linear regression for these interactions with subsequent step-wise backward selection (cut-off value used is the AIC (p=0.157)) to identify a combination of independent factors that most accurately predicts the outcome in this dataset (Table 1).[4] The analysis was performed using R version 3.1.2[5] with the additional rms package.

Variable	**Placebo**	**BMC**	**Main Effect**	**Univ. Interaction**		**Multiv. Interaction**	
	(n=91)	**(n=95)**	**p-val**	**p-val**	β	**p-val**	β
Delta EF (4 months)	3.2	5.5	**0.02**				
Age (years)	56.6	55.4	0.55	**0.04**	- 0.18	**0.05**	- 0.18
Gender (% male)	84.6	81.2	0.52	0.52	+ 1.69		
Weight (kg)	84.3	80.4	0.07	**0.07**	+ 0.12	**0.02**	+ 0.17
BMI	27.6	26.8	0.13	0.26	+ 0.29		
Hypertension (%)	59.3	52.6	0.36	0.51	+ 1.32		
Hyperlip (%)	60.4	51.6	0.23	0.79	- 0.53		
Diabetes (%)	23.1	10.5	0.02	0.64	- 1.33		
Fam. Hist. CAD (%)	36.3	34.7	0.83	0.08	+ 3.6		
Previous MI (%)	6.5	5.2	0.95	0.29	+ 4.7		
Smoking Hist. (%)	68.1	74.7	0.32	0.06	+ 4.1		
Active smoker (%)	42.9	47.4	0.54	0.11	+ 3.2		
Baseline imaging							
EF (%)	47.0	48.3	0.36	**0.02**	- 0.25	**0.002**	- 0.42
ESV (ml)	74.0	67.4	0.12	**0.11**	+ 0.06	**0.08**	- 0.09
EDV (ml)	138.2	128.5	0.12	0.50	+ 0.02		
Intervention							
Days MI-therapy	4.3	4.3	0.66	**0.05**	+ 1.4		
Days MI-BM asp	3.9	3.8	0.60	0.09	+ 1.6		
Basal migr	91.5	103.7	0.22	0.16	-0.02		
SDF stim migr	161.8	170.9	0.49	0.46	-0.008		

Table 1. Baseline characteristics of the REPAIR-AMI trial and interaction modeling of ΔEF with univariable regression analysis and multivariable regression analysis with backwards selection.

The randomization of the REPAIR-AMI study generated comparable groups for our analysis with minor baseline differences (Table 1). The combination of independent predictors for treatment response to cell therapy through interaction was patient age (-0.18%/yr, p = 0.05), weight (+0.17%/kg, p=0.02), $EF_{baseline}$ (0.42%/% p=0.002) and $ESV_{baseline}$ (-0.09%/ml, p=0.08) (Table 1). β-values are expressed as EF change per unit of assessment. These outcomes suggest, that advanced age is associated with poor response to BM-MNC therapy, whereas higher weight, and high initial functional loss are associated with greater treatment benefit in this dataset (Figure 1 A-D).

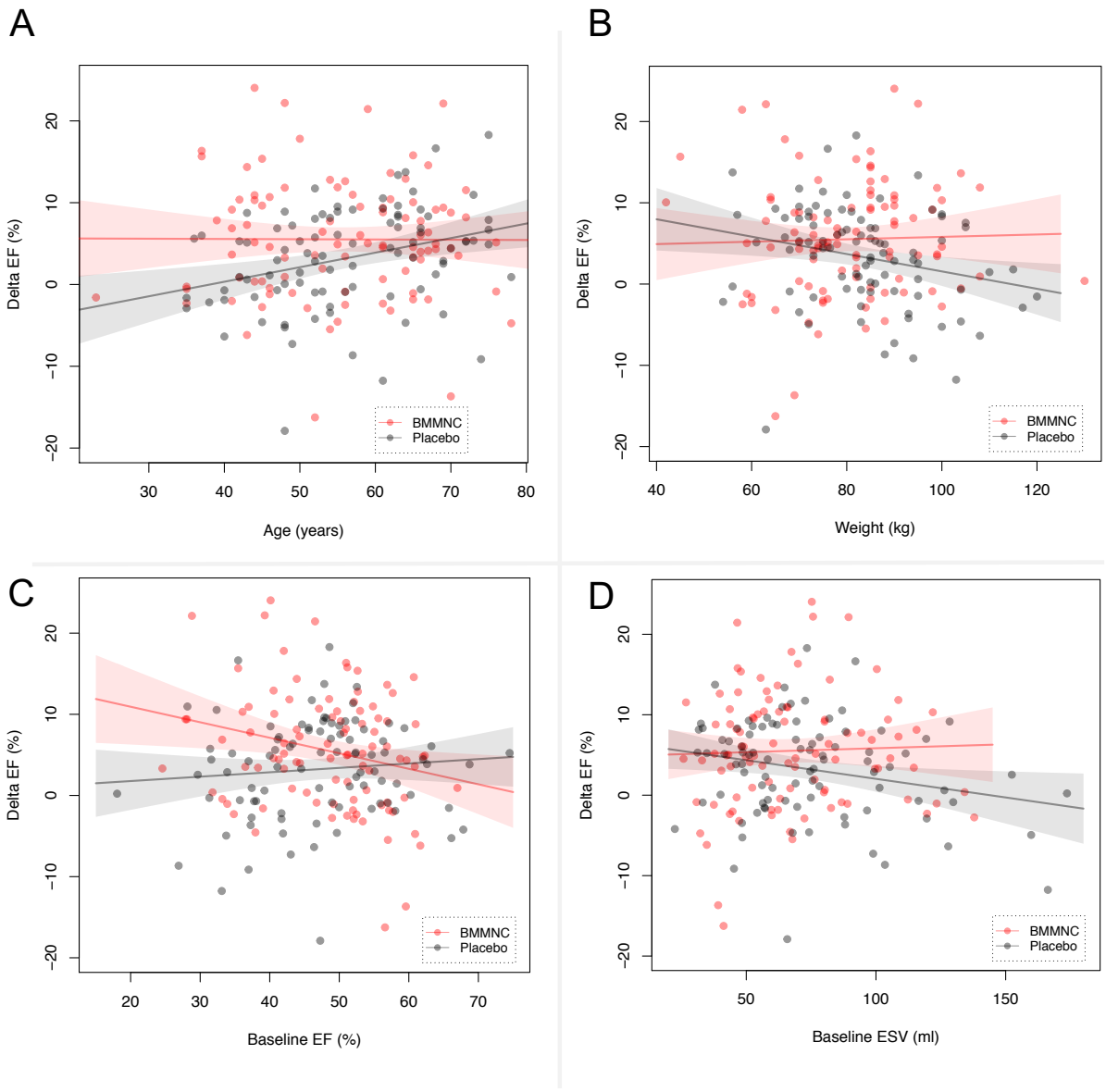

Figure 1. Visualization of the interactions for (A) age, (B) weight, (C) baseline ejection fraction, and (D) baseline end systolic volume.

Discussion

Here, we show the concept of multivariably assessing the benefit of cell therapy by comparing outcomes to a patient's 'reference point' instead of the patient's baseline measurement. Distinguishing responders from non-responders could be a next step for clinical cell therapy, ultimately tailoring cell therapy to patients who will most likely benefit. Statistically correcting for the whimsical nature of the disease is an insightful step in this process. When doing so, it appears that in the REPAIR-AMI trial, younger patients with larger infarcts and risk factors such as smoking and obesity derive more benefit from BMMNC therapy compared to the patients with a negligible risk factor profile. Our findings are partially in line with results from previous meta-analyses, showing more effects of cell therapy in patients with lower baseline EF and age.[1] For the effect of baseline cardiac function and cell therapy, results have been conflicting in both single studies and

meta-analyses, of which a comprehensive overview was recently published.[6] In these meta-analyses the imaging method is also discussed, in which MRI showed less effects compared to the LV angiography used in for example the REPAIR-AMI trial. A recent individual patient meta-analysis could not confirm the findings with regards to stratified variables for age and functional parameters, nor find other associated risk factors with any outcome.[7] Weight as an effect modifier on functional response after cell therapy has never been described before to our knowledge.

Our first multivariable results might imply that cell therapy predominantly affects adverse remodeling after MI. An increased effect of cell therapy with increasing weight is in line with this hypothesis, as waist circumference and BMI are associated with increased incidence of heart failure after MI[8] and increase in bodyweight/BMI is associated with an increased risk of developing heart failure in general.[9] The same holds true for lower baseline EF and developing heart failure after MI.[10] BMMNC therapy might be predominantly counteracting this process through its paracrine mechanisms.
Decreased numbers of circulating progenitor cells have been observed in for example smoking[11], diabetes[12] and obesity[13] and this decrease in circulating cells ultimately leads to worse cardiovascular prognosis.[14] Interestingly, the acute increase in circulating progenitor cells after MI[15] is also diminished with risk factors like diabetes[16] and history of MI.[17] In BMMNC therapy after MI, this defect in progenitor cell mobilization might be partially circumvented by mechanical BMMNC aspiration and subsequent direct administration. It is conceivable however, that patients with few risk factors, and therefore an intact mobilization response, gain little additional benefit from BMMNC treatment. This is also in line with the findings from the CCTRN trials that personal bone marrow characteristics could explain infarct size reduction irrespective of cell therapy in both MI and heart failure.[18,19]

Variability in treatment success in clinical autologous stem cell trails is determined by two factors: the potency of the cell isolate and the disease state of an affected patient. This is in contrast to for instance medicinal therapy, where the variability in treatment response is theoretically solely dependent on the patient, as potency of different drug batches should ideally be nearly identical. Interestingly, the direction of effects from the identified risk factors, almost all besides age, pointed to a greater treatment response with an adverse risk-factor profile. This finding is contrary to results from preclinical studies studying the cell product, which show that cardiovascular risk-factors are associated with poorer pro-angiogenic capacity of human bone marrow cells in preclinical models.[20,21] Pre-clinical studies have heretofore only been able to demonstrate a reduction in the pro-angiogenic potential of the BMMNC graft, but supply little information on the recipient risk factor-fed hearts.

Importantly, the size of the REPAIR-AMI is insufficient to visualize all potential interactions that one might expect, as interaction analyses need more participants to obtain adequate power than analyses of main effects in linear regression models.[22] It is possible that this analysis is incomplete in identifying the effects of biological interactions that predict response in the general population of MI patients and that the found interactions vary in effect size. There are many more (pre)clinical hints from other studies that might also have an effect on the response to cell therapy. Therefore,

the effect of the specific combination of cardiac function, age and weight is applicable to this dataset and should not be applied to patient care yet. This analysis should be seen as a proof-of-concept, and primarily hypothesis generating; the observed (combination of) independent predictors should be confirmed in other datasets and ideally be prospectively declared in larger trials (like the currently recruiting BAMI trial (NCT01569178, www.bami-fp7.eu)) to generate the responder characteristics within the included population. Although adequately powered *a priori* for this analysis, there still is a risk of multiple testing here, as others before us analyzed this dataset in the past. Most importantly, this analysis shows the estimation of a 'true' effect compared to placebo treatment in a trial with a continuous outcome like ejection fraction.

Myocardial infarction and its aftermath can have a capricious course, blurring any effect of therapy to specific subgroups. Identifying responding populations through additional analyses might however be the next step towards optimal cell therapy in clinical care. In this paper, we show a first step in identifying these subgroups using interaction models in a multivariable fashion. Future steps include prediction models for responder identification based on more retrospective and prospective data, to ultimately treat the patients that will benefit most from cell therapy.

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Summary & General Discussion

The results in this thesis have generated interesting responses during many meetings, conferences and peer review. Especially in cardiac development it is crucial to find new strategies to screen for intrinsic and environmental cues for certain processes like cardiac regeneration. Sharing of negative data on certain potential targets is important to prevent the same dead-end projects going over and over. In parallel, we can use systematic review and meta-analysis (MA), to investigate and generate theories that have been on many minds in the field. In our hands, a systematic review and MA is used as both synthesis of available (pre)clinical evidence and a starting point of new hypotheses.
Confirmation through new original research and synthesis of available evidence is as important as novel exploratory original research nowadays; especially since the replication of clinical added value from promising preclinical results is at an all-time low and funding seems to be often wasted in biomedical research, approximated to be a mind-blowing $28 billion per year in 2014.[1-3] In the past years, researchers have also questioned our results and their novelty, claiming to be already fully aware of certain trends and described mechanisms, all carefully studied and shown in this thesis. While there is definitely such a thing as gut feeling, hindsight bias also affects the judgment of proud scientists[4]; everything seems clearer when properly introduced, studied and proven.
Since repetition and assembly of all chapters will increase the chance that conclusions and recommendations from this thesis will 'click and stick', we will summarize our most important findings and will put them in perspective with each other and current literature. Next, we will share our thoughts on relevant future perspectives.

Part 1: Looking back in cardiac development

Cardiac development has served as a welcome starting-point in search of both mechanistic insights in pathology and new therapeutics for cardiac regeneration. By the discovery of the specific pathways and checkpoints responsible for generation and maintenance of cardiac cell types, researchers hold the key to engineer and mature cardiac tissue *in vitro* and potentially activate endogenous dormant pathways in the adult heart.[5] In **Chapter 2** we discussed the importance of Wnt signaling in both cardiac development and disease. We describe that both the presence and absence of Wnt signaling can be beneficial and detrimental in certain stages of cardiac development, as is generally accepted in the process of mesoderm development and later cardiomyogenesis.[6,7] The reactivation of Wnt-signaling in certain cell types upon myocardial infarction (MI) has also been shown by multiple groups, including our own.[8,9] The question remains if there is a true stem cell based on Wnt-signaling and Lgr5-expression, as has been nicely shown over the past year in multiple organs and cancers that (in itself) are known to be more self-proliferative than the adult heart.[10,11] Furthermore, it is all but clear if there is need for agonizing or antagonizing Wnt signaling after MI, as current studies have shown conflicting results, using multiple models of disease and intervening strategies. Of note, the most clinically reliable interventions will obviously be studies using drug-like treatments, started only after disease initiation.[12-15] An inhibitor of Wnt-signaling has been used in a first swine study recently, again showing promising results.[16] In **Chapter 3** we used this same Wnt signaling pathway as a positive control to set up a screen for compounds affecting proliferation of ventricular progenitor cells, using *ex vivo*-cultured cardiac progenitors. Previously, it has been shown that activating Wnt

signaling in an early cardiac progenitor state results in high proliferation rates.[17] A high-throughput protocol like this should be able to discover new compounds and pathways affecting cardiac proliferation that, if conserved in adult cardiomyocytes, might trigger the same process in the adult heart. This setup has been used for a large screen, using a known library of approximately 550 National Institute of Health (NIH) drug approved compounds and kinase inhibitors[18], and has revealed interesting hits and pathways leading to increased early cardiomyocyte proliferation (Buikema, Zwetsloot et al., unpublished data).

In the developing heart we know the importance of key genes like Mesp1, Mef2c, Nkx2.5, Islet1, Tbx5, Gata4 and Hand2, which are spatially present and regulate multiple genes in early processes of cardiogenesis.[19] However, it is unknown if subtle differences in lowly expressed transcription factors also directly affect cardiogenesis. These subtle differences might be enough in these complex processes to drive cell types in certain lineages or regions, making them crucial, but more difficult to identify. For this reason, we used a Quanttrx screening approach[20,21] in **Chapter 4** to assess all murine transcription factors in multiple cell types of the developing heart. Through this screen and multiple validation steps, we identified Hnf4α as a potential new transcription factor in early cardiac ventricular cells. As inhibiting Hnf4α resulted in increased proliferation of these early cardiac cells, we wondered if Hnf4α was also expressed in the adult heart, as this could serve as a potential biologically relevant factor and potential drug-target. We detected Hnf4α in the developing heart, but could not confirm its absolute presence in the adult setting. Although it seemed to be present on a protein level, transcriptional levels were extremely low in adult cardiac tissue, making it unlikely that Hnf4α itself is present and can be maintained on a stable level. Since research is continuously evolving, a state-of-the-art Quanttrx method has been surpassed by techniques like RNA-sequencing. Recent papers from two independent labs showed regional heterogeneity and matching transcriptory signatures for the heart and specific regions during its early development.[22,23] In both datasets, Hnf4α was not detected as an important driver of cardiomyogenesis or regional specificity. Other targets of interest were also not picked up by these papers, such as the expression and importance of Osr1 in the second heart field and outflow tract, which was in our Quanttrx screen and found by others.[24] The other cell types of the heart also did not express Hnf4α to an extent worth mentioning in both papers. This might imply that Hnf4α is only important in earlier stages of cardiac development, for example through cholesterol-derived steroid hormones from the endoderm, as recently shown.[25] As we picked up transcriptional Hnf4α expression primarily in Nkx2.5^{+} cells, it is likely that Hnf4α itself is present in mesodermal progenitors and not only in visceral endodermal layers. This is further supported by recent evidence, in which Hnf4α is also reported in the developing heart, causing congenital heart disease when inhibited.[26] Presence of any Hnf4α-like protein in the adult intercalated disc seems interesting, as much needs to be elucidated in this region. For identification of these proteins, a group from Utrecht artificially amplified isolated intercalated disc protein samples; Hnf4α was not among the identified proteins.[27] If proteins reside in the heart that have an Hnf4α-like similar ligand binding domain, remains to be studied.

Part 2: Looking back in preclinical trials

In **Chapter 6** and **Chapter 7** we report the first systematic review and MA of all placebo-controlled cardiac stem cell studies in preclinical MI models, yielding 80 relevant studies. Striking differences were observed between small and large animal studies in terms of efficacy and quality. In the quality assessments, minor publication bias and attrition bias were observed. In the fields of preclinical research, these biases especially remain one of the challenges nowadays.[28,29] To extend some of these findings to other cell therapy fields, we took multiple systematic reviews on preclinical cell therapy in **Chapter 8** and found common denominators among different diseases related to cell therapy efficacy. Animal size seems to influence efficacy of cell therapy regardless of the disease, while the cell type of the therapeutic also might play a role.
Importantly, the registration of research protocols is an essential tool to make sure that meta-research is conducted in a reproducible and audible way. After revealing the research plan, one can still deviate from this (as we marginally did in **Chapter 7**), but researchers have to have a solid reason for this. Since the number of both clinical and preclinical MA is increasing and studies are of highly variable quality[30,31], it is crucial to put in place more quality controls like the registration of meta-research protocols. Preclinical trials might also be in need of registration upfront, as it would improve quality, reduce publication bias and serve as an extra incentive to publish all studies.[32] The current Experimental Design Assistant initiative by the National Centre for the Replacement, Refinement and Reduction of Animal Research (NC3Rs) is also in line with this philosophy and could potentially aid (and partially replace) trial registration, as it produces a date-stamped protocol for a preclinical study to be conducted.[33] Being able to register a protocol beforehand and provide it with a primary submission means researchers actually can prove that they are reporting what they set out to do. Recent calls for transparent reporting in combination with the ARRIVE guidelines (also developed by the NC3Rs) are other initiatives that aid in the optimal reporting of all necessary data, which is raising standards of preclinical research.[34,35]

In **Chapter 5**, we show the interesting effect of clinically prescribed MI comedication (aspirin, ticagrelor, metoprolol, captopril and atorvastatin) on multiple cardiovascular disease assays, based on a meta-analytic hint and *in vitro* studies on human cells. This has never been studied, yet is likely present in the clinical human situation, while neglected in our preclinical assays. We therefore highly recommend to use these medications in any *in vitro* and *in vivo* assay if possible (see Table 1 for potential dosage strategies *in vivo*). Interestingly, this has been marginally studied before in cell therapy with single drugs added to myoblasts, endothelial progenitor cells or MSCs.[36-38] However, this 'trend' never caught on and nobody has ever tested cellular therapeutics on the background of all clinically prescribed MI drugs. For the sake of simplicity, not all studies should be performed in this drug-controlled setting. However, for confirmatory studies this might be crucial to optimize our pipelines for new potential treatments. This is also supported by our data in **Chapter 9**, where we show that comedication (defined as one or more of clinically prescribed MI drugs) among other methodological variables, reduces infarct size in our large animal models, not even regarding the decrease in efficacy of any therapeutic under study. These first results hint towards an explanation of a significant part of translational failure, which of course is still also comprised of biases, risk factors and other heterogeneity of the clinical disease.[1,39]

	Aspirin	Ticagrelor	Atorvastatin	Metoprolol	Captopril
Human	1 mg/kg	1 mg/kg	.25-.5 mg/kg	2.5 mg/kg	1 mg/kg
Mouse model	10 mg/kg	10 mg/kg	2.5-5 mg/kg	25 mg/kg	10 mg/kg
Pig model	1.1 mg/kg	1.1 mg/kg	.275-5.5 mg/kg	2.75 mg/kg	1.1 mg/kg

Table 1. Suggested adequate oral dosing for common animal models for comedication-correction, based on allometric scaling (assuming no inter-species differences in affinity, distribution and expression).[40]

In multiple chapters, we also took research methodology to a next level. In **Chapter 9** we used a novel approach for studying animal model differences on primary outcome measurements in the field of MI, using only the data from control groups. Through MA, we combined all control groups of ~250 preclinical MI studies, which hypothetically mimic the 'natural course' of that disease model (without any intervention present) and therefore gives us information on model characteristics and standard outcomes. We were able to show that choice of species, sex, MI model, comedication and follow-up duration all independently affect primary outcome measures. We recommend taking these variables into account when designing your study and whenever comparing studies to other literature. Interestingly, researchers might already be aware of these trends in the clinical situation with regards to sex and comedication (also see **Chapter 5**).[41,42] It is comforting to see that clinical heterogeneity also translates to our preclinical models. The need to include both sexes to increase translatability is nowadays also asked for by the NIH and is perfectly in line with the results presented in this thesis.[43]

For confirmatory large animal trials, researchers probably need to conduct their studies in both sexes and control for other variables like comedication, before commencing clinical trials. In the past decades, multiple research fields have formed large consortia and focus groups to tackle new treatment strategies together in the fields of stroke (MULTI-PART), cardioprotection (CAESAR) and cardiac regenerative strategies (TACTICS / ESC working groups).[44-47] Based on the data we have shown on standard primary outcome measures in large animal MI models, it might be wise to standardize animal models in certain research areas, to be able to most accurately compare study results to one another. These consortia also provide the opportunity to test certain therapies in a preclinical multi-center fashion (referred to as 'phase III preclinical studies'), which is also proposed as a new method to increase translatability.[48]

Of note, increasing comparability (meaning using comparable models for multiple studies) might hamper heterogeneity and therefore clinical translatability. In this sense, it is a trade-off between external (heterogeneous) validity and comparability among preclinical studies.

In **Chapter 10**, we took a closer look at the use of standardized mean differences (SMDs) and its use in funnel plots, showing that these outcome measures (in combination with its standard error) are inappropriate for funnel plot analyses. We show these detrimental effects through empirical datasets and illustrative simulations and also provide work-around solutions. This issue is not commonly acknowledged in guidelines or published reports.[49,50] If research groups want to normalize data and are interested in publication bias, we recommend using normalized mean differences or a precision estimate based on study sample size ($1/\sqrt{n}$). This has been neglected for

SMDs, but has been appreciated for odds ratios, which share the same dependence of the standard error on the outcome measure itself.[51] Through recalculation of precision estimates and additional analyses, we show here that collaborators and others have (unknowingly) falsely accused research fields of massive publication bias. Interestingly, these outcome measures are mainly used in preclinical research, as certain absolute values (e.g. in grams, liters or other scores) cannot be directly compared across species or animal models. As the number of preclinical MA is increasing rapidly[31], awareness on this topic seems mandatory.

Part 3: Looking back in clinical trials

In **Chapter 11**, we applied the same approach as in **Chapter 9**, to the clinical setting, looking at control subjects and the influence of sham procedures on standard primary outcomes. Through these analyses we were able to show that delivery procedures might have a biological effect in cell therapy trials, potentially explaining reduced efficacy in (more) properly controlled clinical trials on top of a genuine placebo effect. With regards to sham bone marrow punctures, there does not seem to be an added effect, which was confirmed in chronic kidney disease patients, showing no immediate mobilization of circulating progenitor cells after bone marrow puncture. We have knowingly added to the absurd amount of MA on clinical cell therapy, that at some point outnumbered the amount of properly conducted randomized controlled trials.[52] Due to its specific question and novel approach (focusing on the control patients), we still felt entitled to do this analysis and think this significantly adds to our understanding of cell therapy trials. For the currently including BAMI trial (http://www.bami-fp7.eu/, NCT01569178) this result might be a relief, as their use of standard medical care in the control group will likely not be affected by the absence of bone marrow puncture procedures in this group. However, the procedural effect of the delivery procedure itself cannot be neglected as part of the treatment strategy, regardless of the cell therapy that comes with it.

In **Chapter 12** we tried to unravel responder definitions in the REPAIR-AMI trial, a trial using autologous bone marrow-derived mononuclear cells with continuous outcome measures over time in a capricious disease like MI. By using multivariable interaction modeling, we were able to model response in comparison to (similar) patients receiving placebo. Cardiac function, age and weight together seem to predict who might respond most after cell therapy. If researchers encounter either unhealthy autologous cells, or an MI that can be healed through intrinsic mechanisms, cell therapy might be less advantageous. This suggested correction in prediction strategies is crucial, as primary outcomes like cardiac function and infarct size after MI can differ significantly based on risk factors. This implies that the effect of any intervention over time may differ substantially between patients who are likely to recover from their MI compared to patients who will deteriorate, regardless of any added therapeutic effect. To make matters more complicated, the autologous cellular therapeutic from the bone marrow is also considered to be influenced by many environmental factors.[53-55] Since our field is currently dealing with non-binary outcomes and measurements over time in small groups, this is an enormous challenge. Our developed model now needs proper validation in other datasets to be accurately used in the clinic, for which the first steps have already been taken.

Future perspectives; looking back to think ahead (and move forward)

We live in exciting times in the biomedical sciences. Through the use of new evolving techniques, researchers are generating new insights from multiple 'omics' levels and using techniques to delicately study single cells and their function and expression patterns, through which they are able to interfere and edit genomic material on a cellular level. On the translational level, researchers are applying new regenerative strategies in animal models and are moving to clinical phases rapidly. All these techniques spark new insights, potential therapies and knowledge on deeper levels than we could have ever imagined a decade ago. However, the risk of all this excitement is that researchers currently generate enormous amounts of data and only run off (read: publish) with the most appealing novel pick. I believe we should sometimes grant ourselves time to look back at previous datasets, as there must be incredibly interesting information in these papers, dormant, just waiting to be found. For example, using multiple datasets and finding overlapping patterns for non-cherry picked hits might be a next step in biomedical research. Also, potentially interesting negative findings should be reported, as researchers can save other scientists the time and misplaced enthusiasm if they can properly show that some hypotheses are not what they thought they were. There are multiple initiatives to increase these efforts for negative data, which include dedicated issues from journals[56] to complete new journals focusing on negative results.[57] There has also been a call for so called 'registered reports' (https://osf.io/8mpji/), meaning journals accept projects based on study setups with proper hypotheses, before actual outcomes of the studies are known.[58] Grant programs also seem to chip in, as programs from the United Kingdom's National Institute for Health Research only officially hand over a certain amount of grant money after publication, regardless of the outcome. The Netherlands Organization for Health research and Development (ZonMW) also contributes, having grants to specifically publish neutral and negative findings open access.

The power and risks of preclinical systematic review and MA

Preclinical systematic reviews and MA have emerged as a new tool to analyze preclinical studies. Preclinical MA differ from clinical MA in terms of substantially more heterogeneity, smaller study sample sizes and increased chances of bias. These aspects are both a blessing and a curse; there is more heterogeneity and (biological/methodological) variability to explore, while finding true 'summarizing' values are less of an option; preclinical MA should ideally be used as a quality control of accumulated evidence and for hypothesis generation, rather than pinpointing effects and true means. Interestingly, if we can explore heterogeneity, it does provide us with estimations of directions of effects (see **Chapter 9**), which ideally could serve as more realistic starting points for current preclinical disease models. Defining average mortality (with appropriate confidence intervals), for instance can seriously aid in power calculations for similar preclinical models, for which currently only expert-opinions and experience are used. Since researchers can generate hypotheses through these datasets, it is interesting to see these analyses as a starting point, rather than a summative conclusion, which can be followed by additional hypothesis-driven analyses (this thesis, **Chapter 8** after **Chapter 7**), biological exploration (this thesis, **Chapter 5 & Chapter 11**) or an immediate, adequately powered confirmatory trial.[59] Future state-of-the-art preclinical MA should ideally serve an unanswered question or need and are followed by more than just

summarizing statements, if heterogeneity exploration generates new hypotheses.

Validity of current research in cell therapy

In hindsight, especially the external validity of discussed cell therapy research seems likely to be reduced by:

(1) heterogeneity of the clinical disease itself and the cellular therapeutic (**Chapter 12**), (2) the presence of bias in preclinical cell therapy research (**Chapter 6&7**),

(3) the (non-)heterogeneity of animal models of these diseases (**Chapter 8&9**) and

(4) the (non-)administration of common comedication associated with the disease in preclinical phases (**Chapter 5 and 9**).

Is this a problem? Yes. Is this worrisome? Partially. In hindsight, everything looks clearer and obvious, while researchers in the past have not deliberately modelled their diseases suboptimal. It is the future that holds the truth on the willingness of researchers to avoid biases and study true disease scenarios to a certain extent. The current situation seems not in favor of most effectively translating new therapies to the cardiovascular clinical situation. The optimization of our animal models will likely improve both validity and comparability, hopefully leading to reduction and refinement of animal experiments. Standardization of cardiac large animal models through large consortia like CAESAR and TACTICS will likely benefit validity, comparability and translatability.[45,47] Although preferred, increasing comparability by model standardization must be weighed against a reduction in heterogeneity, which will always be present in the non-standardized clinical disease situation we try to translate to.

The choice of starting first clinical trials on cell therapy also raises new questions with recent acquired knowledge. If (pre)clinical trialists knew what we know today, they might have chosen differently on many aspects (and the start) of these first-in-man studies. Yet, in translational research it is sometimes crucial to move forward and practice, if a therapy has been proven safe and applicable in animal studies, accompanied by promising additional effects. The current system of translational research can be improved, but the mandatory roadmap for any new therapeutic is comprised of so many steps and roadblocks, that putting in more boundaries in the phase of first clinical trials would make new innovations reach the patient even slower. While performing their trials, clinical trialists should always keep open the option of using preclinical models to go back and answer new questions and lasting uncertainties. For this, the cell therapy field serves as a good example, with many preclinical studies on new cellular products and therapy improvements, alongside ongoing clinical trials. These new clinical trials are being conducted in multiple phases and diseases, on the one hand trying to confirm an irrefutable added benefit of the most commonly used cell types (e.g. autologous bone marrow-derived cells) and on the other hand testing next-generation cell types like mesenchymal and cardiac stem cells, combinations of cell types and other modified cellular products in smaller studies.

However, if the large trials fail to show this definite indisputable added benefit, do we put our faith in improved preclinical disease modelling or do we continue to test next-generation therapies in the clinical setting? We will need the leading researchers on cell therapy to discuss these important and potential unpleasant issues, as (temporarily) stopping a departed 'clinical-trial-train' for cell therapy seems rather difficult in a world where added benefit for patients goes hand in hand with scientific development and competition. If we do proceed with clinical trials, we might be better off

with tailoring therapy first and put our new money on improved biological products like next-generation cell products or exosomes.

Evolving therapies need evolving models for evolving disease

As therapies and readouts evolve, it makes sense that the disease under study also changes. In light of better secondary prevention and initial primary interventions during ischemic events, patients are better treated (including pharmacological treatments) and show milder disease despite the presence of multiple risk factors. A changing disease spectrum might call for evolving preclinical models, that most accurately and efficiently can test new therapeutics for added efficacy. This does not mean there is a need to study mild disease only (which will inevitably lead to reduced therapeutic windows), but includes the introduction of guideline-implemented pharmacological treatments or risk factors in preclinical phases to incorporate the existing beneficial/detrimental effects. Evolving disease also implies that MI has a spectrum of 'disease severity', in which an intervention like cell therapy might apply better to some patients than others. Investigating trade-offs between myocardial need for extra repair and therapeutic potency of autologous products, both driven by risk factors and disease severity, might improve and personalize our regenerative strategies.

Better treatment will call for more stringent power calculations and many more included patients to show an effect. If power calculations are based on (too) optimistic preclinical studies, a 'number needed to include' might be artificially low and a first reason that a clinical trial is less likely to show a proper effect. Furthermore, if researchers want to include only patients with severe disease and accompanying large therapeutic windows (for example MI patients with an ejection fraction lower than 40%), they might run into the future problem of low inclusion rates due to disease and therapy evolution. This is not only a problem for the cell therapy field. Recently, the DANISH trial, a large trial on ICD patients in non-ischemic cardiac patients, was not able to show an effect for the use of ICD's, which are being implanted as a class I recommendation in the current guidelines.[60] A proposed explanation again was lower event rates and an evolving disease spectrum due to better regular care. Preparing studies (and power analysis) for the current and future disease might be crucial as therapies (and disease) are evolving at such a rapid pace.

Methodology needs to evolve to keep it up with the field

'Research on research' needs to keep up with the rapid pace of current innovations.[61] Given the increase in preclinical systematic reviews and MA in the last decade, normalized outcome measures and bias assessments for preclinical studies are also being used increasingly.[31] This brings about new opportunities, but also new methodological challenges (**Chapter 10**). It is therefore crucial that 'research on research' is funded and conducted, especially in evolving fields that might not have dealt with certain issues before.[61] The self-cleansing ability of science must stay healthy in order to keep up with the massive publication rates and evolving methodology.

Ideally, we will be able to correct for quality of research and certain other influencing variables in future (meta-)analyses, just like regular confounder analyses in clinical research. Using multivariable approaches, as proposed in this thesis (**Chapter 7-9**), is an interesting option to explore these. One could correct either for binary quality measurements (blinding, randomization), but also (more ideally) for continuous quality scores and methodological variables. Interestingly, while preclinical datasets are more heterogeneous, the study mean values could be considered as

actual representatives of the tightly controlled experimental variables, while clinical trials face the problem of *ecological bias* (meaning that the mean of a population (for example age) has a certain range, and therefore cannot be fully extrapolated to the individual). This same heterogeneity could, if statistically proven, also be used to correct for heterogeneity in funnel plots, which is currently one of the reasons of false-positive funnel plots.[62] The use of SMDs is also a reason for false-positive publication bias assessments, due to the dependence of the variance of the SMD on the outcome itself (**Chapter 10**). Of note, this might also cause problems in the weighing of SMDs for MA, with a potential underestimation of general effect sizes (as lower values will have lower variance and therefore a higher precision estimate). In this case, an n-based precision estimate might also be more appropriate, although there is, of course, a loss of information compared to variance-based estimates. It is an interesting future strategy to weigh all preclinical MA on n's instead of variance, as the number of subjects is less susceptible to biases and unrealistic precision. Multiple imputation to account for unknown variables in preclinical MA datasets might seem less of an option, as multiple imputation in a potentially biased dataset might also come up with biased imputed numbers.

Transitional Translational Research

Evolution is required for survival of entities and usually results in most efficient use of resources. Conducting research in the transitioning landscape of "big team science" nowadays means finding many collaborators, thinking about next steps and always answering meaningful questions, ideally involving some form of valorization. Especially in the fields of biological therapeutics, biologists, engineers, methodologists, (pre)clinical trialists and patients are working in collaborative effort. This phenomenon of collaboration is felt on every level (this thesis, UMC Utrecht's 'Connecting U' strategy, national efforts for joint cardiovascular research (CVON/ICIN/NHI), and international grant applications (NIH, Horizon 2020)). In light of the need to collaborate, quality of research in all phases must increase to make sure that everybody can trust one another and translation takes place as efficient as possible. Teaching both current scientists and scientists of tomorrow seems crucial, to have everybody on the same page. For the training of 'translational scientists' there is no available blueprint yet, although senior translational researchers have acknowledged the need for multimodal translational teaching programs.[63] It seems impossible for any person to master and combine all the skills needed in translational science (epidemiology, statistics, biology, genetics, clinical care, etc)[64], yet it is crucial to have people on teams who bridge research areas, controversies and dogma's by having a solid background in more than one of these. Some doctors should do research, some basic scientists should do comprehensive statistics and some statisticians should see a patient once in a while to increase understanding, cooperation, translation and ultimately the continuous collaboration between research fields. Only by having diverse research teams with broad expertise who are willing to collaborate with other groups, can we most efficiently study new phenomenon, translate these quickly towards other research fields and adequately bridge gaps in cardiac repair and other interests.

Final conclusions and take home message

In this thesis, we were able to look back at different mechanisms and datasets to answer important questions, propose new steps forward and think ahead in the field of cardiac repair. Through this, we have tried to provide new insights on potential therapeutic targets, gave the community necessary feedback, confirmed proposed biological influences in our animal models and gave clear recommendations on improving our translation efforts towards the clinic. Suggested changes will not come by a silver bullet, but more likely through incremental changes in our day-to-day research causing many marginal gains.

Of note, only looking back and thinking ahead will not give us our needed innovations. Generated hypotheses need to be pragmatically tested in both the preclinical and clinical scenarios to not only think ahead, but to actually move forward. This thesis serves as a checkpoint and quality improvement in these efforts, but more importantly the start of new research projects and hypotheses to ultimately provide:

- the best translation for our future stakeholders and collaborators,
- the most accurate efficient models for our research groups and others and
- properly tested innovations to our patients in need

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