Autologous Cell Therapy for Peripheral Arterial Disease

Systematic Review and Meta-Analysis of Randomized, Nonrandomized, and Noncontrolled Studies

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Rationale: **Critical limb ischemia is a life-threatening complication of peripheral arterial disease. In patients who are ineligible for revascularization procedures, there are few therapeutic alternatives, leading to amputations and death.**

Objective: **To provide a systematic review of the literature and a meta-analysis of studies evaluating safety and efficacy of autologous cell therapy for intractable peripheral arterial disease/critical limb ischemia.**

Methods and Results: **We retrieved 19 randomized controlled trials (837 patients), 7 nonrandomized trials (338 patients), and 41 noncontrolled studies (1177 patients). The primary outcome was major amputation. Heterogeneity was high, and publication bias could not be excluded. Despite these limitations, the primary analysis (all randomized controlled trials) showed that cell therapy reduced the risk of amputation by 37%, improved amputation-free survival by 18%, and improved wound healing by 59%, without affecting mortality. Cell therapy significantly increased ankle brachial index, increased transcutaneous oxygen tension, and reduced rest pain. The secondary analysis (all controlled trials; n=1175 patients) shows that there may be potential to avoid ≈1 amputation/year for every 2 patients successfully treated. The tertiary analysis (all studies; n=2332 patients) precisely estimated the changes in ankle brachial index, transcutaneous oxygen tension, rest pain, and walking capacity after cell therapy. Intramuscular implantation appeared more effective than intra-arterial infusion, and mobilized peripheral blood mononuclear cells may outperform bone marrow–mononuclear cells and mesenchymal stem cells. Amputation rate was improved more in trials wherein the prevalence of diabetes mellitus was high. Cell therapy was not associated with severe adverse events. Remarkably, efficacy of cell therapy on all end points was no longer significant in placebo-controlled randomized controlled trials and disappeared in randomized controlled trials with a low risk of bias.**

Conclusions: **Although this meta-analysis highlights the need for more high-quality placebo-controlled trials, equipoise may no longer be guaranteed because autologous cell therapy has the potential to modify the natural history of intractable critical limb ischemia. (*Circ Res*. 2017;120:1326-1340. DOI: 10.1161/CIRCRESAHA.116.309045.)**

Key Words: angiogenesis ■ diabetes mellitus ■ epidemiology ■ mortality ■ regeneration ■ stem cells

Peripheral arterial disease (PAD) is a common complication of atherosclerosis and of rarer systemic diseases, such as thromboangiitis obliterans (or Buerger's disease).[1] In a 2014 US National survey,[2] the prevalence of PAD was found to be in 10.7% of individuals aged ≥40 years. Annually, 11.2% of patients with PAD had critical limb ischemia (CLI), defined as chronic ischemic rest pain, ulcers, or gangrene. In one third of cases, CLI developed without a prior diagnosis of PAD, especially in patients with a history of diabetes mellitus, stroke, heart failure, or renal failure. CLI is associated with poor outcomes, with 1-year amputation and mortality rates of 30% and 25%, respectively.[3] Surgical or percutaneous revascularization is the optimal treatment for CLI,[4,5] which is expected to result in improved limb salvage and survival.[6] Despite a general increase in accessibility to such procedures, still up to 50% of CLI patients are not candidate to revascularization, and long-term mortality remains high.[7] This makes the prognosis of CLI worse than that of several types of cancer.[8] Patients with PAD have a high prevalence of coronary and cerebrovascular disease and up to a 6-fold increased risk of death from coronary artery disease.[9] In fact, mortality in PAD patients is mostly because of cardiovascular causes, but not necessarily related to CLI or direct consequences of limb ischemia.[9]

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Novelty and Significance

What Is Known?

- Peripheral arterial disease (PAD) is a common and severe condition, which when complicated by critical limb ischemia (CLI) could lead to amputation and death.
- Though limb salvage has improved by revascularization, ≤50% of patients with CLI are ineligible for this procedure.
- Bone marrow–derived cells participate in vascular repair, and several clinical trials have been conducted to evaluate the effects of cell therapy on PAD/CLI.

What New Information Does This Article Contribute?

- We present an updated critical review of the literature and meta-analysis of studies evaluating the efficacy of autologous cell therapy for PAD/CLI.
- With an overall low–moderate quality of the evidence, our meta-analysis of randomized controlled trials indicates that cell therapy has the potential to reduce the rate of amputation and improve amputation-free survival and several indices of perfusion.

Previous meta-analyses on cell therapy for PAD/CLI failed to deliver a critical review of the available literature and reached inconsistent conclusions. We elected to meta-analyze randomized, nonrandomized, and noncontrolled trials to gather insights into the evolution of this research field. Our results suggest that cell therapy has the potential to modify the natural history of PAD/CLI by dramatically reducing the amputation rate. Importantly, this finding mostly relies on earlier, lower-quality trials, whereas it is not significant in later, higher quality studies. Instead of concluding that a systematic bias underlies the observed benefits of cell therapy, we provide a detailed discussion of alternative explanations. Subanalyses and meta-regressions highlight critical issues that will need to be covered in future studies.

Nonstandard Abbreviations and Acronyms

ABI	ankle brachial index
BM	bone marrow
CI	confidence interval
CLI	critical limb ischemia
MNC	mononuclear cells
MSC	mesenchymal stem cells
PAD	peripheral arterial disease
PB	peripheral blood
PFWD	pain-free walking distance
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
RCT	randomized controlled trials
RR	risk ratio
TAO	thromboangiitis obliterans
TcO_2	transcutaneous oxygen tension

This clinical and epidemiological scenario shows that the optimal management of PAD is still unmet in a vast number of patients, providing a compelling rationale for the application of advanced therapies against limb ischemia.[10] Since the discovery that blood cells contribute to postnatal angiogenesis,[11,12] there has been a flourishing of clinical studies to test the efficacy of autologous cell therapies for the treatment of CLI, ranging from case reports, small series, uncontrolled trials, and randomized controlled trials (RCTs).[13] Over the years, meta-analyses of such studies have reached inconsistent conclusions on whether cell therapy has beneficial effects on PAD and patient outcomes.[14–20] The pooled analysis of a limited number of placebo-controlled RCTs showed no overall effect,[14] whereas combining high-quality with low-quality RCTs yielded effect estimates in favor of cell therapy.[15,17] Meta-analyses are becoming increasingly used to support evidence-based medicine, but caution should be paid when interpreting their conclusions because of several technical issues.[21] The overall low quality of cell therapy trials is an important concern, but one meta-regression analysis relating reporting errors to effect size in cardiac cell therapy studies has been extensively criticized.[22,23] Nonetheless, meta-analyses are powerful tools in scientific research, allowing to summarize the accumulated evidence and performing exploratory analysis to drive future research.[24] An example of this has been recently shown because meta-analyses have clarified that cell therapy may not affect the outcome when used after an acute myocardial infarction, but can result in a dramatic improvement when used in patients with chronic ischemic heart failure.[25,26]

We herein present a systematic review of the literature and a meta-analysis of cell therapy trials for intractable CLI. We think that, in addition to delivering evidence for clinicians and healthcare providers, digging into these data helps addressing open questions and developing future trends in the field.

Methods

Data Sources and Search Strategy

The protocol of the present meta-analysis (CRD42016050239) was published on the http://www.crd.york.ac.uk/PROSPERO website. This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines.[27] The search string was autologous OR "stem cells" OR "stem cell" OR "bone marrow" OR mobilized OR "cell therapy" OR "cellular therapy" AND PAD OR "peripheral arterial disease" OR CLI OR "critical limb ischemia" OR "lower extremity" OR obliterans AND patients OR patient. The search strategy was first developed in PubMed and then run in ISI Web of Science, Scopus, www.clinicaltrials.gov, and Cochrane Central Register of Controlled Trials. To identify further articles, we hand-searched related citations in retrieved studies, review articles, and commentaries.

Study Selection

For the systematic review, we searched all clinical studies wherein patients with severe intractable PAD or CLI received autologous cell therapy. We extracted case series with at least 8 patients (equal to the

10th percentile of sample size in all retrieved studies), uncontrolled trials, non-RCTs, and RCTs. Eligible studies had to be published in the English literature up to July 2016. The underlying clinical condition described in the eligible studies could be atherosclerosis or thromboangiitis obliterans. Studies with <8 patients, or those using allogeneic cells, or not reporting poolable estimates of efficacy were excluded.

Data Extraction

Two authors (M. Rigato and G.P. Fadini) independently extracted data on the population under study, patient characteristics, type and dose of cell therapy, relevant outcomes, and safety. When disagreement occurred, a third author (M. Monami) was involved to resolve the controversy. The primary outcome was the rate of major amputation (defined as the removal of the limb or a part of it above the ankle) in the cell therapy versus control group. Amputation-free survival, all-cause mortality, and complete wound healing were considered as secondary objective binary end points. Other secondary outcomes were perfusion indexes (ankle brachial index [ABI] and transcutaneous oxygen tension [TcO_2]) and subjective symptoms of ischemia (pain score and pain-free walking distance [PFWD]). If raw data on a specific end point were not directly reported but were obtainable from a graph or figure, data were extracted using GraphClick 3.03. All adverse effects, as well as serious and nonserious adverse events, were recorded to describe safety.

Analytic Strategy

The primary meta-analysis was performed on all RCTs. We then analyzed separately nonrandomized trials, and a secondary analysis was performed on all controlled trials (randomized and nonrandomized). Thereafter, we analyzed separately noncontrolled trials for surrogate end points measured at baseline and end of observation, and a tertiary analysis was done on all trials (controlled and uncontrolled).

Subgroup Analyses

Prespecified subgroup analyses included distinction by study quality and design (randomization and use of placebo), cell product type, route of administration, duration of follow-up, and fixed/random effect model.

Data Synthesis and Statistical Analysis

Continuous data were reported as mean and standard deviation. If the data were reported as median, mean and standard deviation were estimated. In case of missing data or reporting discrepancies, investigators of included studies were contacted by email for clarification and provision of requested data. Because a minority of studies reported intergroup comparisons of changes from baseline in continuous outcome variables (eg, ABI and TcO_2), we calculated the mean difference between groups using values recorded at the end of the observation period. If needed, this could be pooled with the mean difference of changes from baseline values, as recommended by the Cochrane Collaboration,[28] because the distribution of the 2 estimates is expected to be the same. In the absence of patient-level data, this approximation leads to a lower risk of bias as compared with the calculation of the mean difference in change from baseline using average baseline and final data of each group. Rest pain score was normalized to a 0 to 4 scale by proportions.

For dichotomous variables, risk ratios (RR) were calculated for amputation, amputation-free survival, all-cause mortality, and wound healing. Annualized amputation rates were calculated by imputing a linear distribution of events along time in trials with a follow-up of <12 months. As there were no amputations in some trials, annualized amputation rates may be underestimated.

Owing to the intrinsic heterogeneity of cell therapy trials, meta-analyses were performed with a random-effects model. In a sensitivity analysis, we report results of a fixed-effect model only for the primary analysis. The I^2 statistic was used to assess heterogeneity among studies.[29]

An intention-to-treat approach was always applied for the derivation of number of patients (n), which impinges on weight of each pooled study, even when surrogate perfusion outcome variables were measured at the end of observations in a smaller number of patients (because of death or amputation). This is the only way to keep n constant and avoid that weights in the pooled analyses of continuous outcome variables become dependent on amputation-free survival.

Publication bias was assessed by means of funnel plots. The Egger test was used to assess funnel plot asymmetry and publication bias.[30] Sensitivity analyses were run to investigate the associated heterogeneity and the effect of individual studies on it.

Quality Assessment

Two authors independently assessed the methodological quality of the selected studies using the Cochrane risk of bias tool. This scale explores the adequacy of sequence generation, allocation sequence concealment, blinding of participants and caregivers, blinding for outcome assessment, incomplete outcome, selective outcome reporting, and other potential bias.[31] Investigators of included studies were contacted by email when clarification on bias was needed. Any disagreement between reviewers in study inclusion, data extraction, and quality assessment that could not be resolved by consensus were resolved by a third reviewer. All analyses were conducted using the RevMan software.

Trial Sequential Analysis

Trial sequential analysis is a methodology that combines an information size calculation for a meta-analysis with the threshold of statistical significance and allows for quantification of the statistical reliability of data in the cumulative meta-analysis. Traditional meta-analysis runs the risk of random errors because of sparse data and repetitive testing of accumulating data when updating reviews. Therefore, we conducted trial sequential analysis on amputation to calculate the required information size and assess the eventual breach of the cumulative Z-curve of the relevant trial sequential monitoring boundaries for benefit, harm, or futility. We estimated the required information size based on the relative risk reduction point estimate obtained from placebo-controlled trials, with a 80% power and a 2-sided α value of 5%.

Meta-Regression

To explore trial characteristics significantly associated with effect size, we performed meta-regression analyses on all controlled trials. Because there were several study end points and several potential covariates, to avoid an extreme inflation of type I error, a hierarchical strategy was designed to test the mean effect size against RCT characteristics. We first checked for relations between the log RR of the primary end point (amputation) and all variables listed in Table 1. Covariates showing a significant association with the primary end point were then tested for any relation with secondary binary end points. Covariates eventually showing significant associations with secondary end points were then tested for any relation with surrogate end points. The Comprehensive Meta-Analysis software was used to compute and plot meta-regressions.

Results

Included Studies and Pooled Patient Characteristics

Figure 1 reports the flowchart of study search, selection, and inclusion and for meta-analytic strategy. The pooled clinical characteristics of patients, obtained by geometric averages and divided according to study type, are shown in Table 1. Overall, randomized, nonrandomized, and noncontrolled trials included patients with similar characteristics.

Quality of Included Studies

Among RCTs, n=3 were at low risk of bias for all items of quality assessment according to the Cochrane Collaboration tool. For other RCTs, risk of bias was mainly related to random sequence generation, allocation concealment, and blinding (Online Figure I). In several cases, bias was because of

Table 1. Pooled Clinical Characteristics of Patients Included in the Meta-Analysis, Divided According to the Belonging Study Type

Characteristic	All Studies (n=67)	Randomized Controlled Trials (n=19)	Nonrandomized Controlled Trials (N=7)	Noncontrolled Studies (N=41)
Number	2332	837	338	1157
ASO/TAO/unknown, %	59/14/27	67/3/30	62/8/30	55/22/23
Age, y	62.6	65.2	63.4	59.9
Sex male, %	71.9	70.7	72.2	72.9
Hypertension, %	66.1	77.0	63.0	59.9
Diabetes mellitus, %	58.3	61.0	60.2	55.6
Dyslipidemia, %	54.6	76.5	38.6	45.1
Smoke, %	66.6	64.9	66.9	68.0
Chronic kidney disease, %	20.5	26.4	25.5	9.7
CHD, %	38.9	40.5	38.6	37.5
Previous revascularization, %	50.5	51.2	48.1	50.8
Baseline				
ABI	0.49	0.54	0.57	0.47
TcO_2, mm Hg	26.2	36.1	19.5	23.4
Follow-up, months	8.1	6.0	6.8	10.2
Therapies				
Antiplatelet, %	66.8	76.2	83.5	50.5
Statins, %	52.9	81.2	25.7	28.9

ABI indicates ankle brachial index; ASO, atherosclerosis obliterans; CHD, Coronary heart disease; TAO, thromboangiitis obliterans; and TcO_2, transcutaneous oxygen tension.

lack of reporting about procedures for random sequence generation and allocation concealment (unknown risk).

Heterogeneity

Intrinsic heterogeneity among studies was generally high because they differed in setting, underlying disease, type and dose of cells, route of administration, and follow-up duration. On statistical testing, in the primary analysis (RCTs), a significant heterogeneity was noted for amputation-free survival, ABI, TcO_2, rest pain score, and PFWD. In the secondary analysis (all controlled trials), results were the same, but a significant heterogeneity was noted also for wound healing. In the tertiary analysis, heterogeneity was high and significant for all surrogate end points. Accordingly, to compute the pooled RR, we always used the random-effect model.

Publication Bias

In the field of cell therapy, reporting bias is an important issue because negative studies may be filtered, manipulated, or presented in such a way that they become positive, and small negative studies may even remain unpublished. Indeed, according to Egger's test, significant asymmetry was noted for distribution of RCTs in funnel plots of standard error by log RR for amputation and amputation-free survival (Online Figure II). Asymmetry of funnel plots was suggestive of small negative studies being unpublished.

Randomized Controlled Trials

The primary analysis was conducted on n=19 RCTs, including a total of n=837 patients. In these studies, the cell therapy group received one of the following cell product: bone marrow (BM) mononuclear cells (BM-MNCs, n=8 studies) or BM concentrate (n=2 studies), BM mesenchymal stem cells (BM-MSCs, n=3 studies), or mobilized peripheral blood (PB)-MNCs, or

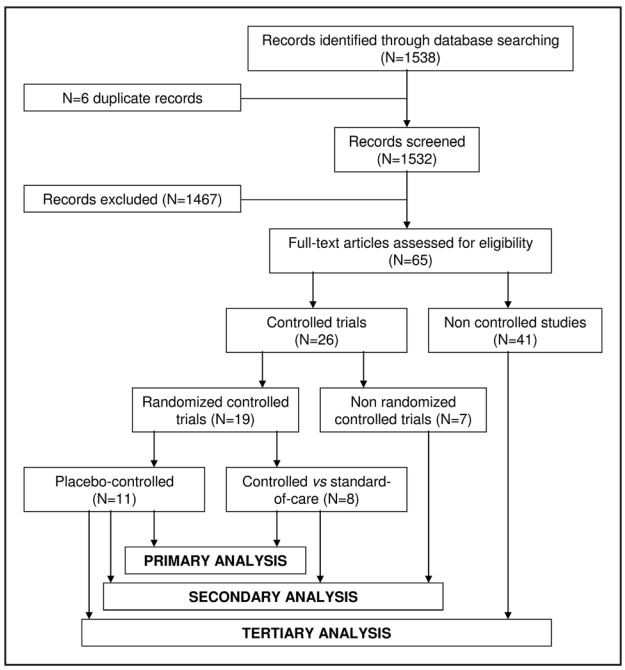

Figure 1. Flowchart and strategy of the meta-analysis.

CD34+ or CD133+ stem cells (n=5 studies); 2 studies used either an ex vivo expanded population of BM-MSCs and macrophages[32] or an ex vivo expanded PB-derived proangiogenic cells.[33] One study compared BM-MNCs versus BM-MSCs with random assignment.[34] The route of administration was intramuscular with multiple injections in the calf muscles (n=16 studies) or intra-arterial (4 studies), with 1 study using both.[35] Patients in the control group received either placebo (mostly saline or vehicle, n=11 studies) or no treatment in addition to standard care (n=8 studies). Despite differences in the active treatments and controls, these studies were pooled in the primary analysis of cell therapy versus control. Subanalyses are presented in Table 2 and discussed below.

For the primary outcome, cell therapy was associated with a significant 37% reduction in amputation rate (RR, 0.63; 95% confidence interval [CI], 0.49–0.82; *P*=0.0004; Figure 2A) and a significant increased probability of amputation-free survival (RR, 1.18; 95% CI, 1.04–1.35; *P*=0.01; Figure 2B), though mortality was not significantly improved (RR, 0.80; 95% CI, 0.48–1.33; *P*=0.39; Figure 2C). Cell therapy significantly increased the probability of complete wound healing by 59% (95% CI, 19%–113%; Figure 2D).

Among surrogate end points (Figure 3), cell therapy significantly improved ABI by 0.11 (95% CI, 0.07–0.15; $P<10^{-5}$), TcO_2 by 10.7 mm Hg (95% CI, 4.9–16.6; *P*=0.0003), and reduced rest pain score by 0.74 (95% CI, 0.36–1.12) over a 0 to 4 scale. No significance difference was noted for PFWD (not shown).

As the random-effect model may relatively overweight small studies, as recommended by Sterne et al,[36] we rerun the primary analysis of RCTs using the fixed-effect model. Even with this method, we found consistent improvements of amputation, amputation-free survival, and wound healing in the cell therapy versus the control group (Table 2).

Secondary Analysis: All Controlled Trials

The search strategy retrieved n=7 nonrandomized controlled trials. In these studies, the control group received placebo (n=1) or no additional treatment (blank, n=6) in a nonrandomized fashion. Cell therapy consisted of BM-MNC (n=3),

Table 2. Sensitivity Subanalyses

		Amputation (RR)	Amputation-Free Serviva (RR)	Death (RR)	Complete Wound Healing (RR)	ABI	TcO_2, mm Hg	Pain Score (0–4)	Pain-Free Walking Distance, m
Study design and quality	Nonrandomized	0.17 (0.08–0.34)	2.12 (1.48–3.03)	0.77 (0.36–1.64)	3.36 (1.13–9.99)	0.15 (0.08–0.21)	20.8 (16.4–25.2)	−2.12 (−3.64 to −0.60)	418.7 (194.3–643.1)
	Randomized versus standard of care	0.47 (0.31–0.71)	1.31 (1.04–1.64)	0.69 (0.26–1.83)	2.05 (1.40–3.02)	0.12 (0.06–0.19)	8.22 (4.27–12.2)	−0.83 (−1.36 to −0.30)	178.2 (128.2–228.3)
	Randomized versus placebo	0.76 (0.55–1.05)	1.10 (0.95–1.35)	0.85 (0.47–1.54)	1.39 (0.98–1.096)	0.11 (0.06–0.16)	11.6 (2.11–21.1)	−0.63 (−1.24 to −0.02)	−71.5 (−183.6 to 40.6)
	Low risk of bias	1.00 (0.64–1.56)	0.99 (0.81–1.21)	0.86 (0.40–1.84)	1.05 (0.61–1.78)	0.09 (−0.05 to 0.22)	3.7 (−3.0 to 10.4)	1.00 (−0.09 to 2.09)	N/A
Route (RCTs)	Intramuscular	0.54 (0.39–0.76)	1.22 (1.05-0.42)	0.76 (0.32–1.79)	1.59 (1.19–2.13)	0.12 (0.08–0.16)	13.5 (7.2–19.8)	−0.74 (−1.14 to −0.33)	N/A
	Intra-arterial	0.86 (0.40–1.88)	1.08 (0.75–1.56)	0.85 (0.45–1.60)	1.30 (0.76–2.21)	0.04 (−0.03 to 0.10)	3.1 (−2.90 to 9.00)	−0.8 (−1.55 to −1.55)	N/A
Cell type (RCTs)	BM-MNCs	0.68 (0.46–1.01)	1.11 (0.93–1.32)	0.83 (0.46–1.49)	1.66 (1.20–2.29)	0.09 (0.04–0.15)	7.57 (0.68–14.5)	−0.75 (−1.40 to −0.09)	N/A
	BM-MSCs	0.43 (0.11–1.72)	1.00 (0.63–1.57)	5.00 (0.27–92.6)	1.42 (0.82–2.46)	0.14 (0.0.8-0.20)	21.8 (16.2–27.4)	−0.59 (−1.43 to 0.24)	173.5 (121.1–225.9)
	PB-MNCs	0.42 (0.23–0.78)	1.62 (1.11–2.34)	0.91 (0.12–6.74)	3.22 (0.57–18.2)	0.11 (0.02–0.21)	12.0 (4.2–19.8)	−0.85 (−1.50 to −0.36)	N/A
Follow-up	≤3 mo	0.49 (0.30–0.79)	1.15 (0.96–1.38)	3.30 (0.14–76.5)	1.91 (1.30–2.81)	0.09 (0.05–0.14)	7.14 (2.12–12.16)	−0.95 (−1.48 to −0.41)	178.24 (128.19–228.29)
	6–9 mo	0.82 (0.55–1.20)	1.13 (0.94–1.36)	0.85 (0.48–1.53)	1.47 (0.99–2.19)	0.11 (0.06–0.17)	14.24 (4.05–24.44)	−0.57 (−1.38 to 0.23)	−71.50 (−183.64 to 40.63)
	≥12 mo	0.56 (0.34–0.91)	2.31 (1.05–5.05)	0.53 (0.18–1.61)	1.75 (0.20–15.4)	0.15 (−0.07 to 0.37)	10.10 (0.39–19.81)	−0.35 (−1.43 to 0.73)	93.73 (−30.05 to 217.51)
Analysis	Random (RCTs)	0.63 (0.49–0.82)	1.18 (1.04–1.35)	0.80 (0.48–1.33)	1.59 (1.19–2.13)	0.11 (0.07–0.15)	10.7 (4.9–16.6)	−0.74 (−1.12 to −0.36)	97.3 (−30.1 to 217.5)
	Fixed (RCTs)	0.58 (0.45–0.75)	1.21 (1.10–1.33)	0.81 (0.50–1.32)	1.73 (1.39–2.16)	0.10 (0.08–0.12)	11.7 (9.12–14.2)	−0.76 (−0.9 to−0.62)	123.7 (79.4–168.0)

ABI indicates ankle brachial index; BM, bone marrow; MNC, mononuclear cell; MSC, mesenchymal stem cell; PB, peripheral blood; RCT, randomized controlled trial; RR, risk ratio; and TcO2, transcutaneous oxygen tension.

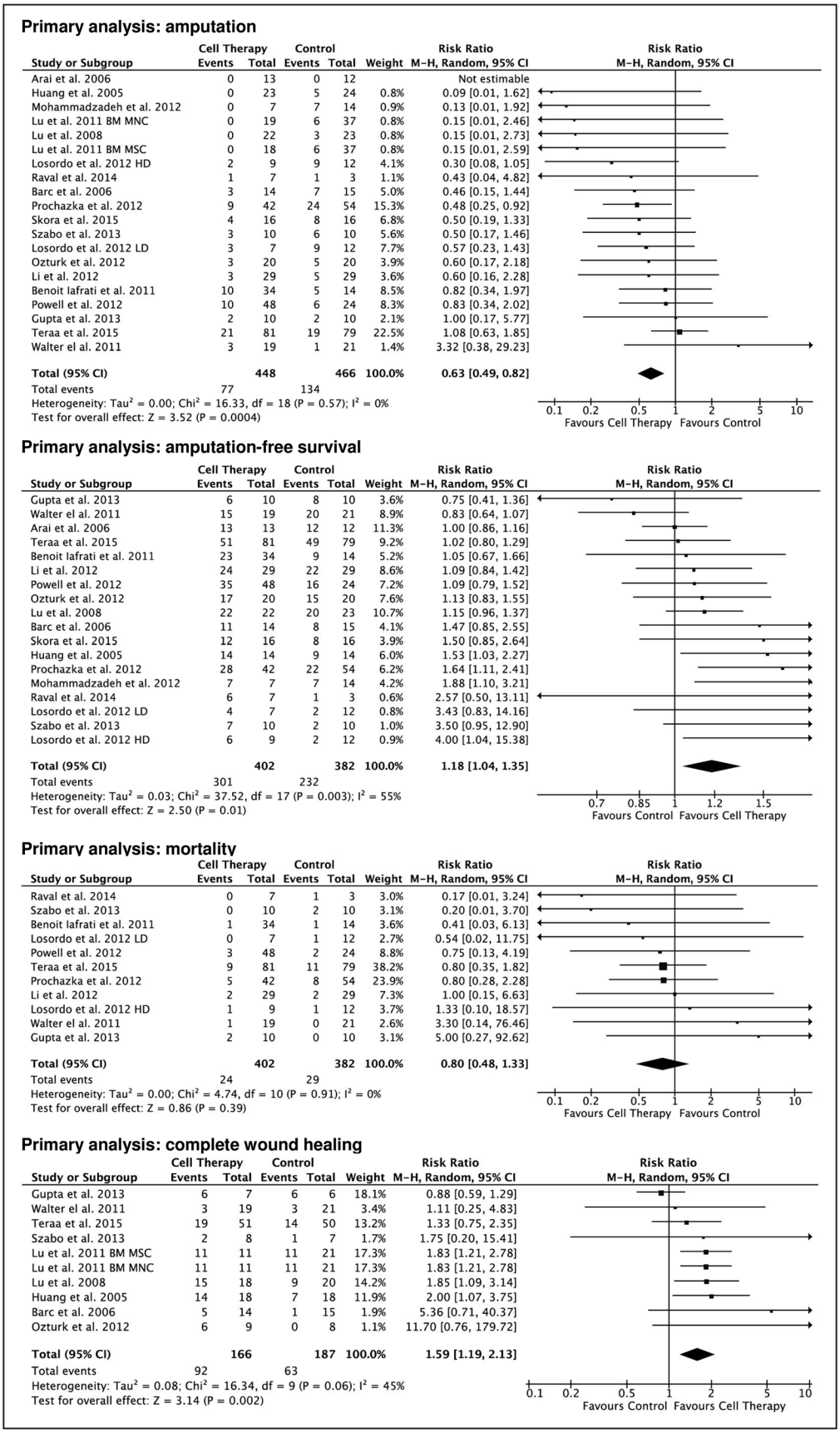

Figure 2. Results of the primary analysis of randomized controlled trials (RCTs) on objective binary end points. Trials wherein mortality could not be estimated have been omitted. Risk ratios and 95% confidence interval (CI) are shown on *x* axis.

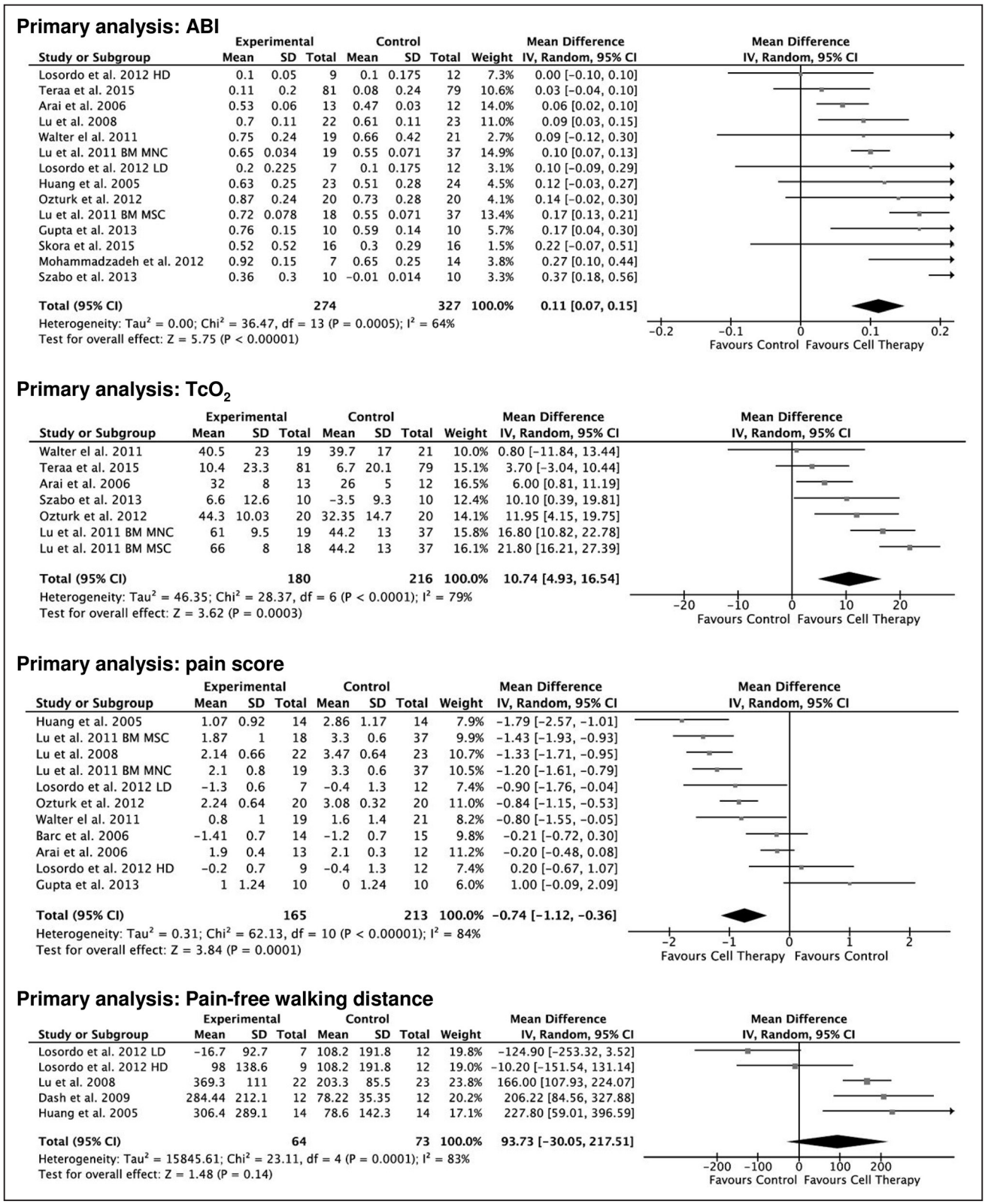

Figure 3. Results of the primary analysis of randomized controlled trials (RCTs) on surrogate end points. Changes in ankle brachial index (ABI; absolute value), transcutaneous oxygen tension (TcO_2; mm Hg), pain (0–4 scale), and pain-free walking distance (m) are shown on the *x* axis, along with 95% confidence interval (CI).

unfractioned BM cells (n=2), PB-MNC, or progenitors (n=3). A pooled analysis of these studies indicate that cell therapy significantly reduced amputation rate by 83% and improved amputation-free survival by 112%, though no significant reduction in mortality was observed. In nonrandomized controlled trials, there were significant improvements in ABI,

TcO_2, pain score, and PFWD in the cell therapy versus control group that tended to be larger than in RCTs (Online Figure III).

To adjust significance levels for sparse data and repetitive testing on accumulating data, the trial sequential analysis was performed as previously described.[37] Online Figure IV shows the plot for the primary outcome (major amputation), indicating that sample size in the primary analysis may be insufficient to exclude false-positive conclusions. Therefore, we run an analysis wherein all controlled trials (randomized and nonrandomized) were pooled together, including 1175 patients: cell therapy significantly reduced amputation rate by one half (RR, 0.48; 95% CI, 0.35–0.66). The annualized amputation rate in all controlled trials was 70.8% in the control group and 27.2% in the cell therapy group (P=0.0002). According to this estimate, the number needed to treat was 2.3. Cell therapy also improved amputation-free survival (RR, 1.40; 95% CI, 1.18–1.65) and likelihood of complete wound healing (RR, 1.67; 95% CI, 1.24–2.25). Surrogate end points of perfusion (ABI and TcO_2) and pain (rest pain score and PFWD) were all significantly improved by cell therapy (Online Figure III).

Tertiary Analysis: All Studies Reporting Quantitative Outcomes

Noncontrolled studies (n=41), which included a total of 1157 patients, were also recorded and analyzed. The cell product was BM-MNCs (n=28), PB-MNCs (n=10), selected cell populations ($CD34^+$, $CD133^+$, or endothelial progenitor cells; n=4), or BM-MSCs (n=1), with 2 studies using both BM-MNCs and PB-MNCs.

Improvement in surrogate indexes of perfusion and pain was evaluated as change from baseline (Online Figure V). These studies cumulatively indicate that after cell therapy, ABI increased by 0.15 (95% CI, 0.11–0.18), TcO_2 increased by 14.1 mm Hg (95% CI, 11.1–17.0), rest pain score decreased by 1.68 (95% CI, 1.44–1.91) on a 0 to 4 scale, and PFWD increased by 259.1 m (95% CI, 182.2–335.9). The annualized amputation rate in these noncontrolled trials was 21.8%, which was similar to that observed in the cell therapy group of controlled trials (P=0.39).

Because these data show benefits of cell therapy that were similar but quantitatively larger than in controlled trials, we also performed a tertiary analysis wherein all studies (controlled and not-controlled) were pooled to estimate the change from baseline induced by cell therapy in surrogate end point measures. Results of this analysis, conducted on n=2334 patients, are shown in Online Figure IV.

Subanalyses of RCTs

Trial Quality

In a subanalysis wherein only randomized placebo-controlled trials were included (n=11), cell therapy was associated with nonsignificant improvements in amputation rate (RR, 0.77; 95% CI, 0.56–1.07; P=0.12), amputation-free survival (RR, 1.10; 95% CI, 0.90–1.33; P=0.36), and wound healing (RR, 1.39; 95% CI, 0.98–1.96; P=0.07). There were still significant improvements in ABI (0.11; 95% CI, 0.06–0.16), TcO_2 (11.6 mm Hg; 95% CI, 2.1–21.1), and rest pain score (reduction by 0.63; 95% CI, 0.02–1.24). When the analysis was further restricted to RCTs with a low risk of bias (n≤3, depending on the outcome), cell therapy appeared to confer no benefit for all end points (Table 2 and Figure 4).

Route of Administration

In RCTs, the most common route of cell therapy administration was intramuscular (n=15), while n=3 trials used intra-arterial infusion and 1 used alternative intramuscular or intra-arterial administration.[35] In a separate analysis for delivery route, only intramuscular but not intra-arterial administration was associated with a significant improvement in amputation rate, amputation-free survival, complete wound healing, ABI, and TcO_2. Rest pain score was significantly improved when cell therapy was administered via either the intramuscular or intra-arterial route (Table 2 and Online Figure VI). In a direct comparative trial, Klepanec et al[38] randomly assigned 41 no option patients with Rutherford stage 5 to 6 PAD to intramuscular or intra-arterial delivery of BM-MNCs: there were no differences between groups in terms of limb salvage and wound healing (>70%), as well as surrogate indexes of perfusion, pain, and quality of life. In another comparative trial, Van Tongeren et al[39] randomly assigned 21 PAD patients to receive intramuscular or intramuscular plus intra-arterial administration of unfractioned BM cells: amputation rate was nonsignificantly lower in the combined intramuscular plus intra-arterial group than in the intramuscular group (25% versus 64%; P=0.17), and surrogate indexes of perfusion improved similarly in the 2 groups.

Cell Product Type

In RCTs, the cell therapy consisted of BM-MNCs, BM-MSCs, or PB-MNCs. Other selected cell types were highly heterogeneous and could not be pooled into a single group. In separate analyses, cell therapy with PB-MNCs, but not other cell types, was associated with a significant improvement in amputation and amputation-free survival, whereas only BM-MNCs significantly improved wound healing (Table 2). Both BM and PB-MNCs significantly improved ABI, TcO_2, and rest pain score. BM-MSCs only improved ABI, TcO_2, and PFWD, despite the previous observation that in vitro and in animal models, neovascularization capacity of MSCs from CLI patients is preserved.[40] In a direct comparative trial, Huang et al[41] randomly assigned 150 PAD patients to BM- or PB-MNC therapy, though only a per-protocol analysis was reported: amputation rate was low and not significantly different between the 2 groups (5.3% in the PB-MNC group versus 8.1% in the BM-MNC group). Improvement in ABI and rest pain was significantly better with PB- than with BM-MNC therapy.[41] Onodera et al, while reanalyzing patient-level data of 2 previous cohorts,[42,43] also reported no difference in amputation-free survival between patients who received BM-MNCs (20.0%) and those who received mobilized PB-MNCs (25.6%).[44] In the substudy B by Tateishi-Yuyama,[45] n=22 patients with bilateral CLI received intramuscular implantation of BM-MNCs in 1 leg and PB-MNCs in the other leg according to a random assignment: local therapy with BM-MNC was superior to that with PB-MNCs in improving ABI, TcO_2, rest pain, and PFWD. In another direct comparative study, Lu et al[34] randomly assigned 41 diabetic CLI patients with ulcers to receive

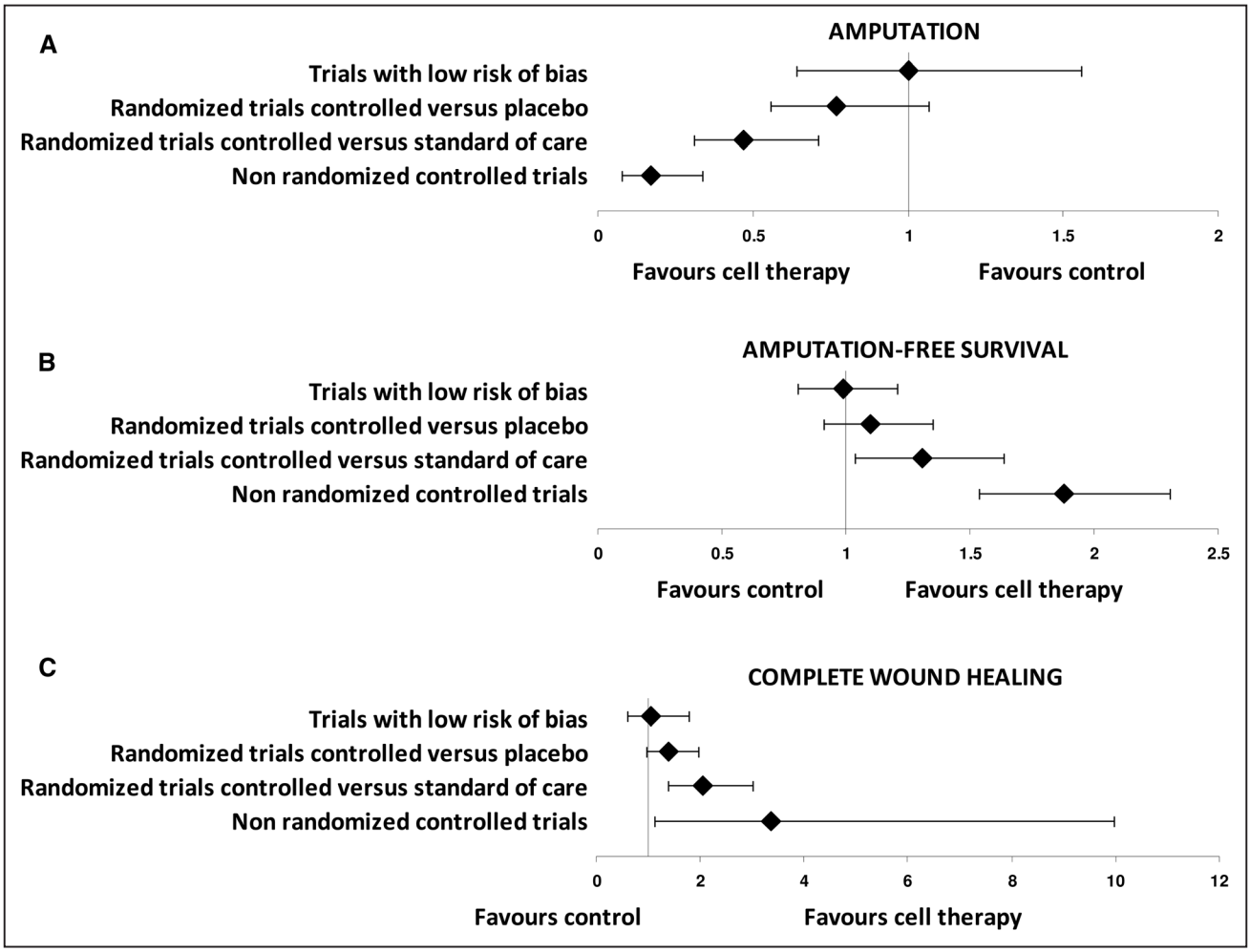

Figure 4. Subanalysis by study design and quality. Outcomes showing significant improvements in primary and secondary analysis were evaluated according to study design (nonrandomized, randomized versus standard of care, or randomized versus placebo) and trial quality. Risk ratio (RR) and 95% confidence interval (CI) are plotted for amputation (**A**), amputation-free survival (**B**), and complete wound healing (**C**).

intramuscular implantation of BM-MNCs or BM-MSCs: BM-MSC therapy was more effective than BM-MNC therapy in improving wound healing and perfusion indexes. These data indicate that direct comparative trials do not consistently show superiority of one cell type over another.

Follow-Up Duration

RCTs were divided into 3 groups according to follow-up duration: (1) ≤3 months; (2) >3 but <12 months; (3) ≥12 months. No consistent trend was detected for any end point according to follow-up duration.

Meta-Regression Analyses

Using the hierarchical meta-regression strategy described in the Method section and illustrated in Figure 5A, we detected an inverse significant correlation between the prevalence of diabetes mellitus and the log RR for amputation in each trial (r=–0.0139±0.0058; P=0.017; Figure 5B), implying that the benefit of cell therapy on amputation rate was higher in trials with a majority of patients having diabetes mellitus. Down on the hierarchical scale, prevalence of diabetes mellitus was not associated with secondary binary end points.

No significant correlation was detected between the primary outcome and duration of follow-up, suggesting that the effect of cell therapy on amputation was not significantly attenuated with longer observation time. No significant correlation was detected between the primary outcome and frequency of use of concomitant medications, such as statins and antiplatelet agents, in each trial.

When all trials were considered, we found no relation between total cell dose or $CD34^+$ percentage and the primary outcome. This negative finding was probably related to heterogeneity in cell type and dose reporting among trials. Indeed, when only trials using BM-MNCs were considered (n=11), we found a significant inverse relation between MNC dose and log RR for the primary end point (r=–0.6±0.2/10^3; P=0.0041; Figure 5C), implying that higher cell doses may exert more beneficial effects on amputation risk. Meta-regressions for other cell types were not performed owing to the small number of studies available. However, concerning PB-MNCs, in a direct cell dose titration trial, Losordo et al[46] randomly assigned n=28 PAD patients to intramuscular administration of low-dose (10^5) or high-dose (10^6) mobilized PB-$CD34^+$ cells or placebo: at 12 months, amputation rate

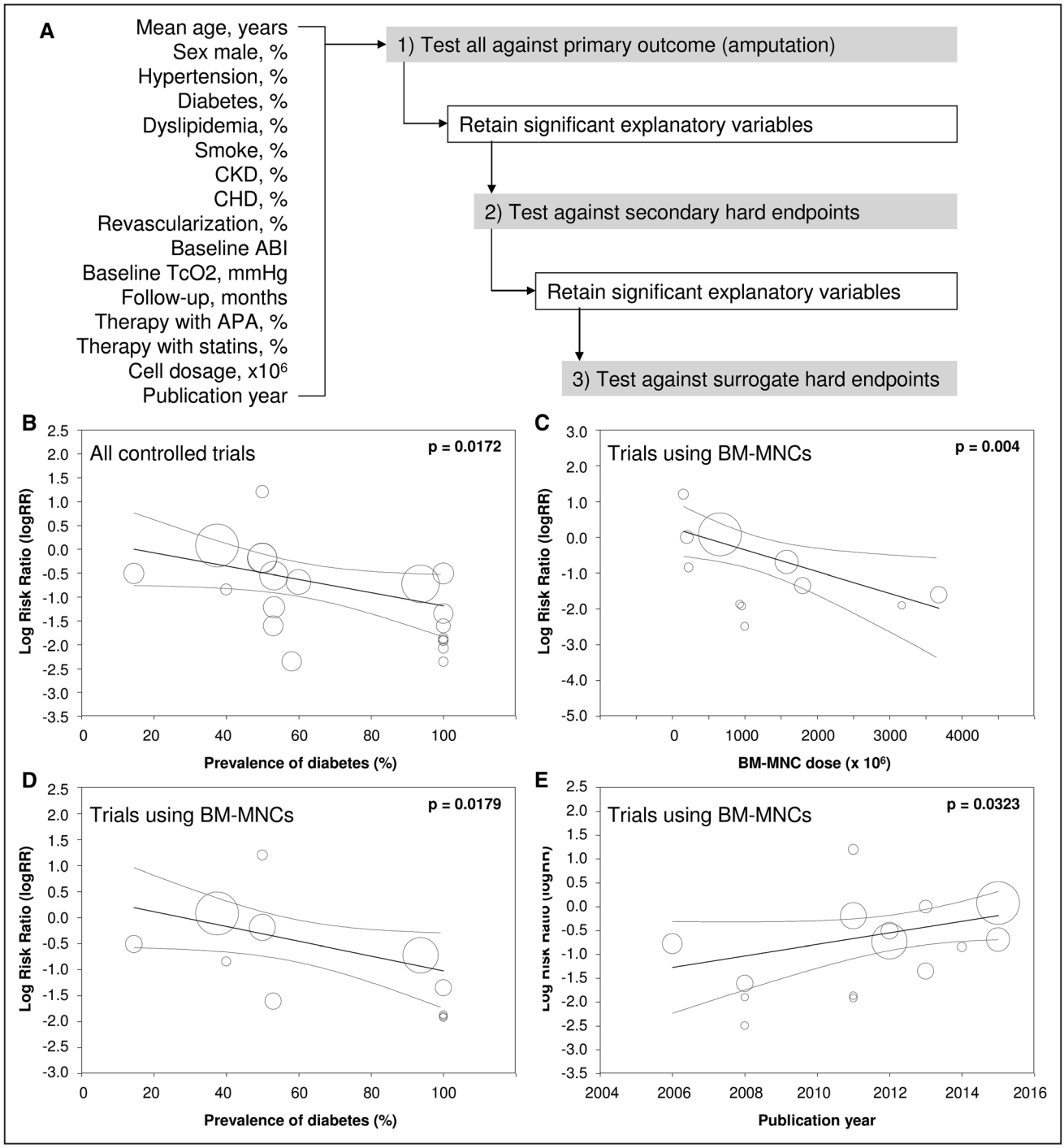

Figure 5. Meta-regression analyses. The hierarchical strategy used to perform meta-regressions is shown in **A**. Meta-regressions were performed first on all controlled trials (**B**) and then on the more subgroup of trials using BM-MNCs (**C**–**E**). ABI indicates ankle brachial index; APA, anti-platelet agents; BM, bone marrow; CHD, coronary heart disease; CKD, chronic kidney disease; MNC, mononuclear cell; and TcO_2, transcutaneous oxygen tension.

was 22% (2/9) in the high-dose group versus 43% (3/7) in the low-dose group (P=0.59). No significant differences between the 2 groups were detected in other end points.[46] Most retrieved studies used a single cell product, which was administered once through a selected delivery route. In 2 nonrandomized controlled trials, patients received 2 consecutive infusions of BM cells done 45 days apart into the femoral arteries.[47,48] To specifically evaluate the benefit or repeating cell therapy over time, Molavi et al[49] randomized n=22 patients with CLI to receive a single or 4 repeated intramuscular injections of BM-MNCs: ABI and pain indexes improved similarly in the 2 groups, but the repeated dose group showed a significantly better improvement in PFWD at 24 weeks.

In the more homogeneous group of trials using BM-MNCs, the meta-correlation with prevalence of diabetes mellitus was confirmed, and we also detected a correlation with publication

year, showing that the benefit of cell therapy on amputation risk in more recent trials was lower than that in earlier trials.

Safety

Among all controlled trials included in the secondary analysis, n=19 studies reported safety outcomes, but reporting was highly heterogeneous and frequently inconsistent. The number of events/patient per year was calculated based on reported events and follow-up duration in each trial and grouped according to the Medical Dictionary for Regulatory Activities system (Figure 6). This pooled analysis indicates that, on average, most patients (80%–90%) are expected to experience 1 nonsevere adverse event during 1 year of observation after cell therapy. As compared with control, cell therapy was associated with a significantly higher rate of all nonsevere adverse events and a nonsignificant increase in nonfatal PAD-unrelated severe adverse events. Death and PAD-related events were not considered in the safety analysis because they represented efficacy end points. The increase in the rate of nonsevere adverse events in the cell therapy group was mostly attributable to injection site reactions and musculoskeletal disorders, whereas other events (such as renal and urinary disorders, nervous system, and psychiatric disorders, as well as metabolism and nutrition disorders) were overall rarer and less frequent in the cell therapy than in the control group.

Discussion

Main Findings

Although there are several important limitations related to included trials and their data, this meta-analysis shows that in PAD/CLI patients who were ineligible for surgical or percutaneous revascularization, autologous cell therapy may have the potential to reduce the risk of major amputation (−36% in the primary analysis) and improve the probability of wound healing (+59%). Though all-cause mortality was unaffected, cell therapy was cumulatively found to improve significantly the chances of amputation-free survival (by 18% in the primary analysis). In addition, cell therapy appeared to ameliorate several surrogate end points of limb perfusion, pain, and functional capacity, as compared with control treatment. Results of the primary analysis, conducted on RCTs, were confirmed and strengthened in the secondary analysis, wherein all controlled trials were included, showing that cell therapy may reduce the risk of amputation by 50% and increase the probability of amputation-free survival by 33% and of wound healing by 67%. Potential improvements in continuous surrogate end points were further confirmed in the tertiary analyses, wherein uncontrolled studies were added, providing data on a total of 2332 patients. We recognize that some uncontrolled studies may have been missed by our search strategy, but the accrued sample size seems sufficient to reach reliable and statistically robust estimates.

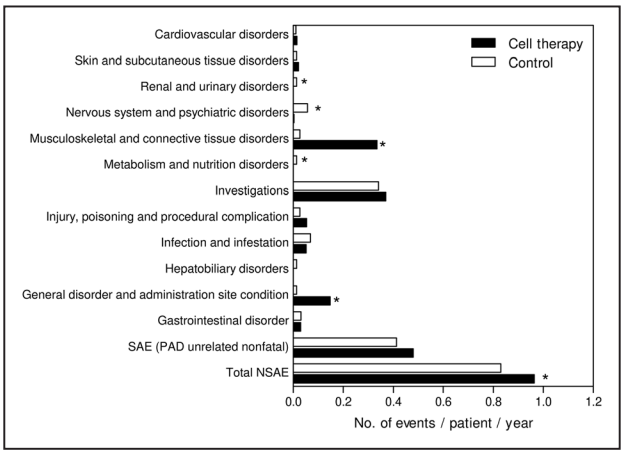

Figure 6. Adverse events in cell therapy trials according to the Medical Dictionary for Regulatory Activities (MedDRA) classification. *P<0.05 for the cell therapy versus the control group, after adjusting for multiple testing using Bonferroni correction. NSAE, nonsevere adverse events; PAD, peripheral arterial disease; and SAE, severe adverse events.

To our knowledge, this is the most updated and comprehensive meta-analysis of cell therapy for PAD/CLI reported to date. Our decision to analyze randomized, nonrandomized, and noncontrolled trials relies on the concept that low-quality studies must be interpreted in view of high-quality studies and vice versa. For instance, the analysis of annualized amputation rates shows that uncontrolled trials already provided good estimates of what the benefit of cell therapy could be in RCTs.

Improvements in amputation and wound healing rates suggest that cell therapy may be able to modify the natural history of intractable CLI. Interestingly, Giles et al[50] reported similar amputation-free survival in a cohort of CLI patients treated with BM cells when compared with a cohort of patients who underwent infrainguinal bypass thought to be at high risk for graft failure. Although this trial was not randomized, it suggests that cell therapy may represent an initial alternative to high-risk infrainguinal bypass.

In our meta-analysis, reduction in amputation rates was not associated with prolonged survival. The lack of effect of cell therapy on overall mortality was expected because the causes of death in patients with severe PAD or CLI are mostly unrelated to PAD.

Overall, cell therapy was found to be safe, being associated with mild and mostly transient adverse events related to local implantation/infusion. Based on these findings and taking into account that up to 50% of CLI patients are not candidate to revascularization, autologous cell therapy may be considered as a new standard of care.

Limitations and Critical Considerations

The enthusiasm of the latter statement must be tapered in view of the limitations of this meta-analysis, namely low–moderate quality, high heterogeneity, publication bias, and possible lack of statistical power. Analyzing the impact of study design is important because the decision and timing for amputation may be subjective, indicating that trials on CLI having amputation as the primary outcome should be double-blind.[3] Results of a subanalysis wherein studies were divided according to design and quality are particularly impressive. For all outcomes, the benefit of cell therapy versus control progressively declined moving from nonrandomized controlled trials to randomized controlled trials versus standard of care, and to randomized controlled trials versus placebo, and finally disappeared in RCTs with a low risk of bias. In addition, a direct correlation was noted between RCT quality (Cochrane 0–6 item scale) and log RR for amputation (P=0.03), implying that higher quality studies yielded less efficacy results.

This observation, partly reported before,[14,17] may suggest that trials lacking randomization, concealing, or blinding were strongly and systematically biased in favor of cell therapy. Taken as such, this finding implies that strength and quality of the evidence is too low to support the use of cell therapy in CLI, and further high-quality trials are needed. This is the conclusion reached by other meta-analyses on this topic. We opine that this reasoning has at least 2 fundamental drawbacks.

First, relating trial quality to effect size can be misleading if, as it happens here, low quality relies on reporting bias, such as a lack of reporting about random sequence generation and concealment, especially in early and small trials. In other terms, the fact that an article does not report on random sequence generation and concealment does not necessarily imply that methods for random sequence generation were biased or that the sequence was not appropriately concealed during the trial. Unfortunately, our attempts to gather missing data from the authors were mostly unsuccessful. To make a parallel, in 2014, a weighted meta-regression analysis found that the number of reporting errors in trials of cell therapy for heart disease was inversely correlated to effect size on ejection fraction.[22] This ecological association was warmly criticized and proven as basically fallacious because it was trying to establish an unlikely causal relationship.[23] In fact, there are several noncausal alternative explanations for the lack of efficacy in higher quality studies. For instance, meta-regression shows that early studies using BM-MNCs reported larger effects than does more recent studies. Instead of being attributable to trial quality, this trend may result from changes in clinical practice over time or improvements in revascularization access and success. A refinement in endovascular and surgical techniques leading to improved limb salvage is expected to reduce the potential incremental benefit of cell therapy. As a consequence, if new RCTs aim to demonstrate efficacy of cell therapy, they will need to enroll larger numbers of patients in a more advanced disease stage. In fact, substantial benefit of cell therapy may be seen only in higher-risk patients, and these may be preferentially included in early, small studies.[51] Preview results of the MOBILE trial (MarrowStim PAD Kit for the Treatment of Critical Limb Ischemia in Subjects With Severe Peripheral Arterial Disease; NCT01049919), wherein 152 CLI patients were randomized to intramuscular implantation of a BM cell concentrate or placebo, support the concept that patients in a more advanced disease stage are those who may benefit most from cell therapy because amputation-free survival was improved only in Rutherford stage-5 patients.[52] A similar conclusion can be drawn from preview results of the PACE trial (Patients With Intermittent Claudication Injected With ALDH Bright Cells; NCT01774097), wherein 82 patients with intermittent claudication were randomized to receive BM-derived aldehyde dehydrogenase bright cells or placebo[53] and showing that collateral vessels increased only in patients with complete femoral artery occlusion.[54] It should also be carefully noted that the definition of intractable CLI varies substantially across countries and even across different sites in the same country, mainly based on endovascular and surgical expertise as well as more or less extensive use of high-risk procedures of limb salvage. In multicenter studies, this is likely to generate a degree of patient heterogeneity that can negatively impinge on the efficacy of cell therapy. Finally, the intervention may have been implemented differently in larger and later studies, resulting in smaller effect estimates.[55] For instance, the quest for the best cell type often led investigators to use new cell products and different preparation techniques, with dosages varying by a factor of 10 or 100, a heterogeneity that can contribute to mask the true effect of cell therapy.

The second important point of discussion is that there is no alternative to amputation in patients with intractable CLI, but cell therapy has the potential to modify the natural history of this life-threatening condition. This has a biological rationale backed by 2 decades of research in cardiovascular regenerative medicine.[56] Therefore, based on our analysis of the literature, even with a low–moderate quality of evidence, one can argue that further RCTs may not be ethical, and these patients should receive cell therapy, where available. In this case, safety and efficacy, along with comparative assessment of the best cell type, dose, and route, could continue to be scrutinized within prospective multicenter observational registries.

The trial sequential analysis suggested that significant pooled effects in RCTs may be false-positive because the required sample size was not reached. To cope with this, we performed a secondary analyses for all controlled trials, which yielded more robust conclusions. The GRADE (Grading of Recommendations Assessment, Development and Evaluation) working group accepts the possibility to upgrade nonrandomized control trials to the moderate quality of evidence,[24,57] but we recognize that this approach is unconventional because it pools studies with different designs. Therefore, results of the secondary analysis should be taken with caution.

Meta-Regressions and Subanalyses

Previous studies suggested that the efficacy of cell therapy may differ in patients with atherosclerosis and in those with thromboangiitis obliterans.[20] We could not perform such subanalysis because of missing data on the underlying cause of CLI in several trials. Nor could we assess whether disease stage had any effect on the benefit of cell therapy because most studies included patients with Leriche-Fontaine stage III-IV PAD, without reporting the proportion of the 2.

In meta-regressions, we found that trials with a high prevalence of diabetes mellitus showed larger benefits of cell therapy on amputation rates. We would like to underline that any attempt to identify predictors of response to therapy are intrinsically biased by the ecological nature of meta-regression.[23] Nonetheless, this finding is biologically plausible because diabetes mellitus impairs BM stem cell mobilization induced by tissue ischemia and reduces homing of cells to damaged tissues,[58,59] thus, making the rationale for intramuscular BM cell therapy. Despite dysfunction of BM cells has been extensively documented in diabetes mellitus,[60] this meta-regression finding suggests that circumventing impaired mobilization and homing may be more important than reversing intrinsic cell dysfunction. However, opposite results were reached in a study designed to detect prognostic factors after PB-MNC therapy, wherein diabetes mellitus had a negative impact on the effects of cell therapy.[61] Furthermore, the preview report of the MOBILE trial (NCT01049919) shows that the benefit of cell therapy on amputation-free survival was limited to nondiabetic patients.[52]

The literature consistently identifies dialytic therapy among negative predictors of efficacy of cell therapy.[43,44] A meta-regression for prevalence of chronic kidney disease or dialysis could not be performed because of the small number of studies reporting detailed information on this comorbidity.

No other meta-regression satisfied our prespecified hierarchical strategy, but reporting heterogeneity likely impinged on significance of meta-regression. For instance, the impact of disease stage on response to therapy may be masked by inability to uniformly record disease stage from trial reports.[62] Similarly, the impact of cell dose was only evident among trials using BM-MNCs because it was impossible to compare doses of total BM-MNC with those of PB-MNCs or more selected populations.

Our subanalyses suggest that intramuscular implantation may be preferable to intra-arterial infusion and that mobilized PB-MNCs may outperform BM-MNCs and BM-MSCs. The lack of efficacy of intra-arterial cell therapy (resulting from a limited number of studies) may be expected in patients with significant stenosis of leg arteries, which would prevent distal delivery of the cells via a scarce blood flow. Rather, an eventual superiority of PB- over BM-MNC therapy should be judged in view of the fact that patients implanted with mobilized PB cells also received G(M)-CSF (granulocyte (macrophage) colony stimulation factor), which may itself affect the outcome.[20] However, a few direct comparative trials show no consistent difference between routes of administration or cell product type. In addition, no clear advantage emerged from selecting specific populations of stem/progenitor cells, such as $CD34^+$ or $CD133^+$.

Finally, we think that the impact of repeated administration of cell therapy on PAD/CLI outcomes should be dissected in future trials, possibly with the support of preclinical studies, as done for cardiac cell therapy.[63]

Regulatory Implications

Exploratory subanalyses imply that intramuscular implantation of BM-MNCs or mobilized PB-MNCs should be considered the standard cell therapy for intractable CLI. Remarkably, this type of therapy, which requires neither sophisticated cell selection systems nor a cath-laboratory, is within the reach of most hub hospitals in developed countries.[64] Cheap automated cell processing systems have been developed to be used at the patient's bedside or in the operating room.[65,66] Regulatory hurdles still limit widespread diffusion of cell therapy, as the European Medicines Agency (EMA) claims that BM-MNCs implanted intramuscularly for CLI is nonhomologous, thereby classifying them as an advanced medicinal product.[67] This position, which restricts BM-MNC preparation to good manufacturing practice–certified facilities, disregards 2 decades of research in cardiovascular regenerative medicine showing the physiological role of BM cells in aiding repair of the vascular system.[56] We expect that such accepted textbook notion, together with results of the present meta-analysis, will drive a change in EMA policy regarding cell therapy for PAD/CLI.

Conclusions

Our primary analysis on RCTs seems to provide conclusive results on the efficacy of cell therapy on several objective and surrogate end points in patients with intractable CLI. Subanalyses for trial design and quality cast doubts on the validity of such findings, suggesting that low-quality studies may have been biased in favor of cell therapy. Therefore, based on a traditional meta-analytic approach, we should conclude that more high-quality RCTs are needed to confirm or definitely exclude ability of cell therapy to improve the outcome of intractable CLI. However, as discussed earlier, there are several noncausal explanations for the unexpected trend shown in Figure 4, which needs to be critically reviewed against several confounders. The risk of incautiously dismissing a potentially effective therapy needs to be weighted against severity of a disease burdened by high morbidity and mortality rates. Therefore, we argue that the scientific community should interrogate on whether we still need additional evidence on this therapy, or we should recognize that cell therapy has the potential to modify the natural history of intractable CLI. If this is the case, equipoise may not be granted in future RCTs.

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Disclosures

None.

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Autologous Cell Therapy for Peripheral Arterial Disease: Systematic Review and Meta-Analysis of Randomized, Nonrandomized, and Noncontrolled Studies

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ONLINE DATA SUPPLEMENT

Items included in this data supplement

Supplemental Table I. Characteristics of included studies. ASO: arteriosclerosis obliterans, TAO: thromboangiitis obliterans, PSS: progressive systemic sclerosis, BM MNC: bone marrow mononuclear cells, BM MSC: bone marrow mesenchimal stem cells, ABMSC: autologous bone Marrow stem cells, EPC: endothelial progenitor cells, IM: intra muscular, IA: intra arterial, IU: intra ulcer, LD: low dose, HD: high dose, AFS: amputation free survival, UH: ulcer healing, ABI: ankle brachial index, TcPO2: transcutaneous oxygen pressure, RPS: rest pain score, PFWD: pain free walking distance. Blank control treatment denotes standard of care. *limb vs limb comparison.

Study, date, ref.	Country	Cause of PAD/CLI	Disease stage	N° of patients (Treated)	Type of cell therapy	Control	Amount of injected cells (10^6)	Route	Follow-up (months)	Endpoints
RCTs										
Arai 2006 [1]	Japan	N.S.	Fontaine III-IV	25 (13)	BM MNC	Blank	1000-3000	IM	1	ABI, TcPO2, RPS
Barc 2006 [2]	Poland	N.S.	Fontaine III-IV	29 (14)	BM MNC	Blank	N.S.	IM IA	6	Amputation Death, AFS, UH
Benoit 2011 [3]	US	ASO	Rutherford 4-5	48 (34)	BMAC	Placebo	N.S	IM	6	Amputation Death, AFS
Dash 2009 [4]	India	ASO (6) TAO (18)	Fontaine II-IV	24 (12)	BM MSC	Blank	7.26 (ASO) 5.04 (TAO)	IM	3	PFWD
Gupta 2013 [5]	India	N.S.	Rutherford 4-6	20 (10)	BM MNC	Placebo	200	IM	6	Amputation Death, AFS UH, ABI, RPS
Huang 2005 [6]	China	ASO	Fontaine III-IV	28 (14)	PB MNC	Blank	3000	IM	3	Amputation Death, AFS UH, ABI, RPS, PFWD
Li 2012 [7]	China	ASO	Fontaine III-IV	58 (29)	BM MNC	Placebo	N.S.	IM	6	Amputation Death, AFS
Losordo 2012 [8]	US	ASO (27) TAO (1)	Rutherford 4-5	28 (16)	PB $CD34^+$	Placebo	0.1/Kg (LD) 1/Kg (HD)	IM	12	Amputation Death, AFS ABI, RPS, PFWD
Lu 2008 [9]	China	ASO	Fontaine II-IV	45 (22)	BM MSC	Blank	732-5600	IM	3	Amputation Death, AFS UH, ABI,

										RPS, PFWD
Lu 2011 [10]	China	ASO	Fontaine II-IV	41 (37)*	BM MSC (20) BM MNC (21)	Placebo	930 (BM MSC) 960 (BM MNC)	IM	6	Amputation UH, ABI, TcPO2, RPS, PFWD
Mohammadzadeh 2012 [11]	Iran	ASO	Fontaine III-IV	21 (7)	PB MNC	Placebo	900-1200	IM	3	Amputation Death, AFS, ABI
Ozturk 2012 [12]	Turkey	N.S.	Fontaine III-IV	40 (20)	PB MNC	Blank	992-1240	IM	3	Amputation Death, AFS, UH, ABI, TcPO2, RPS, PFWD
Powell 2012 [13]	US	N.S.	Fontaine III-IV	72 (48)	Ixmyelocel-T	Placebo	35-295	IM	12	Amputation Death, AFS
Prochazka 2012 [14]	Czech	ASO	Fontaine IV	96 (42)	ABMSC	Blank	N.S.	IA	4	Amputation Death, AFS
Raval 2014 [15]	US	N.S.	Fontaine III-IV	10 (7)	PB $CD133^{+}$	Placebo	50-400	IM	12	Amputation Death, AFS
Skora 2015 [16]	Poland	N.S.	Fontaine IV	32 (16)	BM MNC	Blank	770-3830	IM	3	Amputation Death, AFS, ABI
Szabo 2013 [17]	Israel	N.S.	Fontaine III-IV	20 (10)	Ves-Cell	Blank	66.4	IM	22.6	Amputation Death, AFS, UH, ABI, TcPO2
Teraa 2015 [18]	Netherlands	ASO	Fontaine IIb-IV	160 (81)	BM MNC	Placebo	657	IA	6	Amputation Death, AFS, UH, ABI, TcPO2
Walter 2011 [19]	Germany	ASO (32) TAO (8)	Rutherford 4-6	40 (19)	BM MNC	Placebo	153	IA	3	Amputation Death, AFS, UH, ABI, TcPO2, RPS
Non-randomized										
Bartsch 2007	Germany	ASO	Fontaine	25 (13)	BM MNC	Blank	N.S.	IM+IA	13.1	ABI, PFWD

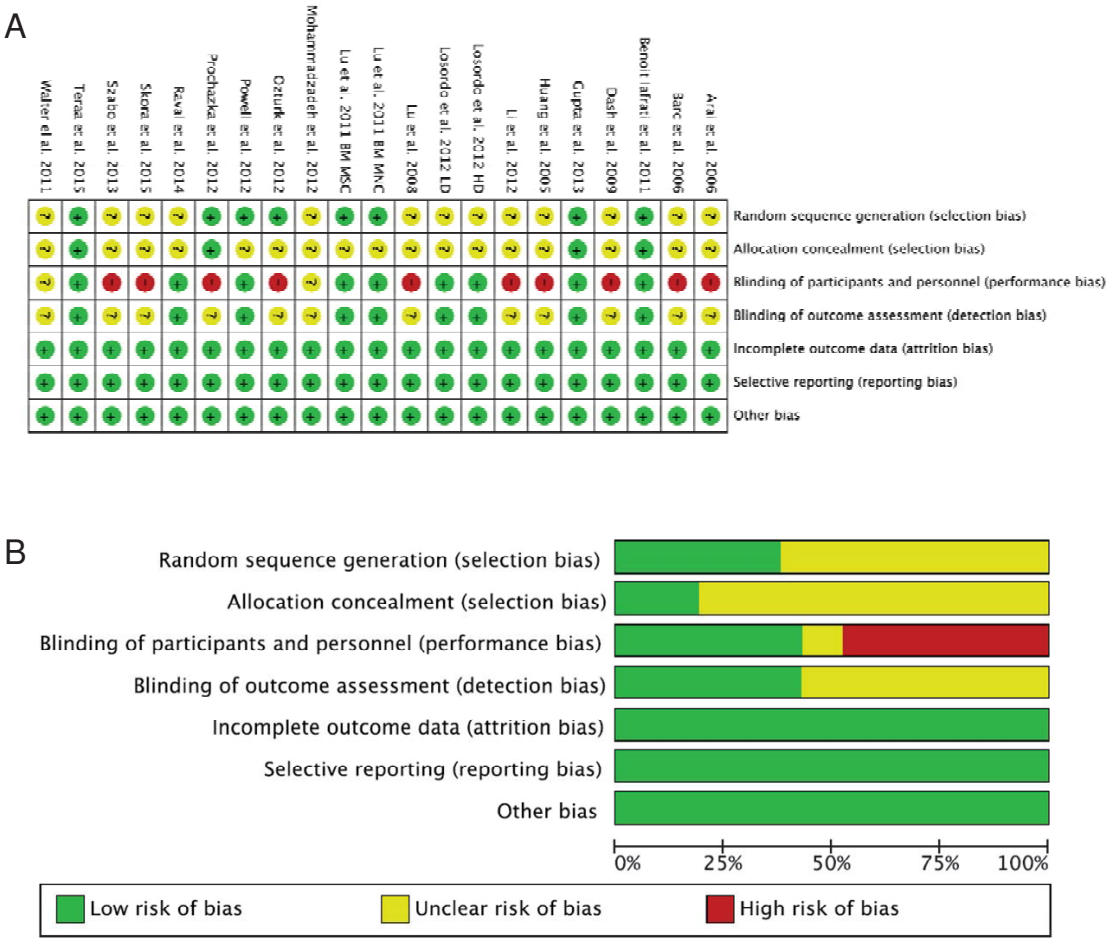
[20]			IIb							
Cobellis 2008 [21]	Italy	N.S.	Fontaine III-IV	19 (10)	BM MNC	Blank	1000	IA	12	Amputation Death, AFS, ABI, PFWD
De Angelis 2015[22]	Italy	ASO	Fontaine IV	86 (43)	PB MNC	Blank	125.6	IM	4.5	Amputation Death, AFS, RPS
Dubsky 2013 [23]	Czech	ASO	Rutherford 4-6	50 (28)	BM MNC (17) PB MNC (11)	Blank	1800 (BM MNC) 10400 (PB MNC)	IM	6	Amputation Death, AFS, UH, TcPO2
Idei 2011 [24]	Japan	ASO (25) TAO (26)	Fontaine III-IV	97 (51)	BM MNC	Blank	1800	IM	57.6	Death, AFS
Napoli 2008 [25]	Italy	N.S.	Fontaine III-IV	36 (18)	BMC	Blank	1310-6030	IA	12	Amputation Death, AFS, RPS
Tateishi-Yuyama 2002 A [26]	Japan	ASO	Fontaine III-IV	25 (24)*	PB MNC	Placebo	1700	IM	6	ABI, TcPO2, RPS
Non-controlled										
Amann 2009 [27]	Germany	N.S.	Rutherford 4-6	51	BM MNC (12) BM TNC (39)	N.C.	1100 (BM MNC) 3000 (BM TNC)	IM	13.7	ABI, TcPO2
Burt 2010 [28]	US	ASO (7) TAO (2)	Rest pain, ABI <0.8	9	CD 133^{+}	N.C.	82.5	IM	12	ABI, PFWD
Chocola 2007 [29]	Czech	ASO (21) TAO (3)	Fontaine III-IV	24	BM MNC	N.C.	53100	IA	12	ABI, TcPO2
Das 2013 [30]	Malaysia	N.S.	Fontaine III-IV	8	BM MSC	N.C.	2/Kg	IA	6	ABI, TcPO2, RPS
Durdu 2006 [31]	Tukey	TAO	Fontaine III-IV	28	PB MNC	N.C.	1690	IM	16.6	ABI
Franz 2015 [32]	US	ASO (74) TAO (2)	Fontaine III-IV	49	BM MNC	N.C.	N.S.	IM+IA	3	ABI
Fujita 2014 [33]	Japan	ASO (4)	Fontaine	11	CD 34^{+}	N.C.	64	IM	13	ABI, TcPO2,

		TAO (7)	III-IV							RPS, PFWD
Gabr 2011 [34]	Egypt	N.S.	Fontaine III-IV	20	BM MNC	N.C.	1110	IM	3	ABI
Giles 2015[35]	Lebanon	N.S.	Fontaine III-IV	20	BM MNC	N.C.	N.S.	IM	17.3	ABI
Heo 2016 [36]	Korea	TAO	Fontaine III-IV	37	BM MNC	N.C.	570	IM	6	ABI, RPS
Huang 2007 [37]	China	ASO	Fontaine	150	BM MNC (74) PB MNC (76)	N.C.	575 (BM MNC) 7201 (PB MNC)	IM	3	ABI, TcPO2, RPS, PFWD
Ismail 2014 [38]	Egypt	N.S.	Fontaine III-IV	20	BM MNC	N.C.	100	UI	36	ABI, RPS, PFWD
Kinoshita-Kawamoto 2012 [39]	Japan	ASO (5) TAO (12)	Rutherford 4-6	17	CD 34^{+}	N.C.	0.5/Kg	IM	52	ABI, TcPO2, PFWD
Kirana 2012 [40]	Germany	ASO	Fontaine III-IV	12	BM MNC	N.C.	306.8	IM	13	ABI, TcPO2
Kolvenbach[41] 2010	Germany	N.S.	Rutherford 4-6	8	BM MNC	N.C.	17.2	IM	9.2	ABI
Lara-Hernandez 2010 [42]	Spain	ASO (26) TAO (2)	Fontaine III-IV	28	EPC	N.C.	N.S.	IM	14.7	ABI, RPS
Klepanec 2012[43]	Slovakia	ASO	Rutherford 5-6	41	BM MNC	N.C.	N.S.	IM (21) IA (20)	6	ABI, TcPO2, RPS
Lasala 2012 [44]	US	N.S.	Rutherford 4-6	26	BM MNC	N.C.	N.S.	IM	4	ABI
Malyar 2015 [45]	Germany	ASO (14) TAO (2)	Fontaine IIb-IV	16	BM MNC	N.C.	420	IM	6	ABI, TcPO2
Miyamoto 2004 [46]	Japan	ASO (6) TAO (5) PSS (1)	Fontaine IV	12	BM MNC	N.C.	4030	IM	1	ABI, RPS
Miyamoto 2006 [47]	Japan	TAO	Fontaine III-IV	8	BM MNC	N.C.	3500	IM	22.8	ABI, RPS
Mizuno 2010	Japan	ASO	Fontaine IV	8	BM MNC	N.C.	N.S.	IM	12	ABI, RPS

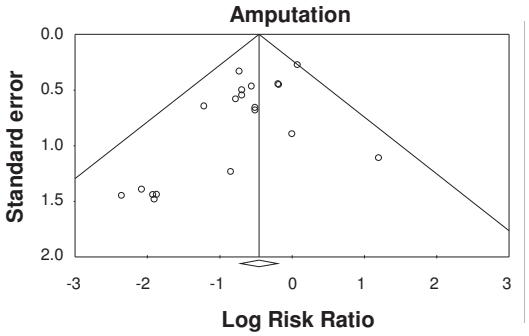
[48]										
Molavi 2016 [49]	Iran	ASO (5) TAO (17)	Fontaine III-IV	22	BM MNC	N.C.	860 (LD) 3720 (HD)	IM	6	ABI, RPS, PFWD
Moriya 2009 [50]	Japan	ASO (28) TAO (14)	Fontaine III-IV	42	PB MNC	N.C.	1500	IM	24	RPS
Motukuru 2008 [51]	India	TAO	Fontaine III-IV	36	BM MNC	N.C.	580	IM	6	ABI, TcPO2, RPS
Murphy 2011 [52]	US	ASO (22) TAO (7)	Fontaine III-IV	29	BM MNC	N.C.	170	IM	12	ABI, TcPO2
Nishida 2011 [53]	Japan	ASO (7) TAO (2)	Fontaine III-IV	11	PB MNC	N.C.	11000	IM	24	ABI
Perin 2011 [54]	US	N.S.	Rutherford 4-5	10	BM MNC	N.C.	1300	IM	3	ABI, TcPO2
Ruiz-Salmeron 2011 [55]	Spain	ASO	Fontaine III-IV	20	BM MNC	N.C.	266.2	IA	3	ABI, TcPO2
Saigawa 2004 [56]	Japan	ASO	Fontaine III-IV	8	BM MNC	N.C.	60.4	IM	1	ABI, TcPO2
Schiavetta 2012 [57]	Italy	N.S.	Rutherford 3-5	34	BM MNC	N.C.	903	IA	12	TcPO2
Skora 2013 [58]	Poland	ASO	Fontaine II-IV	16	BM MNC + VEGF plasmid	N.C.	1580	IM	3	ABI, RPS
Takagi 2011 [59]	Japan	ASO (12) TAO (3)	Fontaine III-IV	15	BM MNC	N.C.	N.S.	IM	1	ABI, TcPO2, RPS
Tateishi-Yuyama 2002 B[26]	Japan	N.S.	Fontaine III-IV	22*	BM MNC (22) PB MNC (22)	N.C.	1500	IM	6	ABI, TcPO2
Van Tongeren 2008 [60]	Netherlands	N.S.	Fontaine III-IV	27	BM MNC	N.C.	1230	IM (12) IA+IM (15)	12	ABI, PFWD
Vriese 2008 [61]	Belgium	ASO	Fontaine III-IV	16	BM MNC	N.C.	1300	IM	3	ABI, RPS
Wan 2016 [62]	China	TAO	Fontaine	64	PB MNC	N.C.	N.S.	IM	28.5	ABI, TcPO2,

			III-IV							RPS, PFWD
Wang 2014[63]	China	N.S.	Rutherford 4-6	25	PB MNC	N.C.	N.S.	IM	4	ABI, TcPO2, RPS
Zhang 2008[64]	China	ASO (10) TAO (5)	Fontaine II-IV	15	PB MNC	N.C.	1787	IM	12	TcPO2, RPS, PFWD
Xu 2015 [65]	China	ASO	Fontaine III-IV	127	PB MNC	N.C.	N.S.	IM	1	ABI, RPS

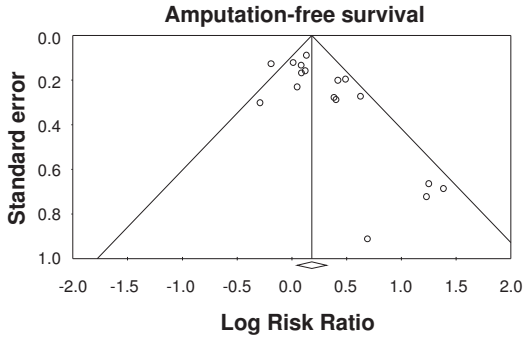
Supplemental Figure I. Quality of RCTs according to the Cochrane Collaboration Manual. A) Detailed item-by-item analysis of study quality. B) Summary of RCT quality showing the percentage of RCTs satisfying each quality item.



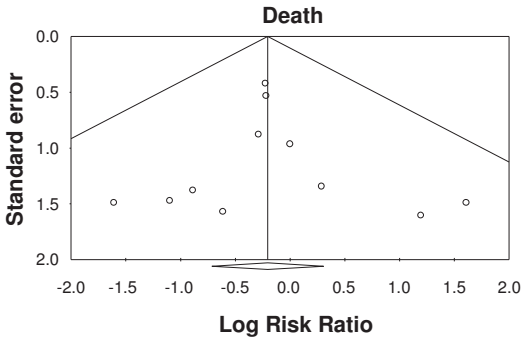
Supplemental Figure II. Funnel plots of RCTs for objective binary end-points.



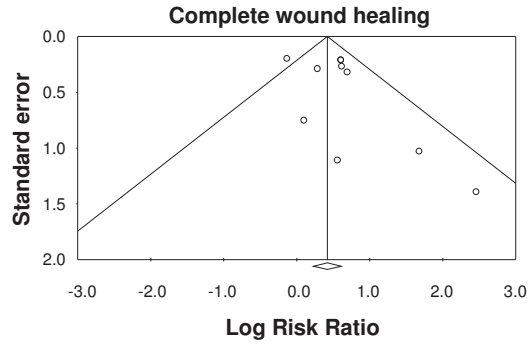
(Egger's test p = 0.016)



(Egger's test p = 0.008)

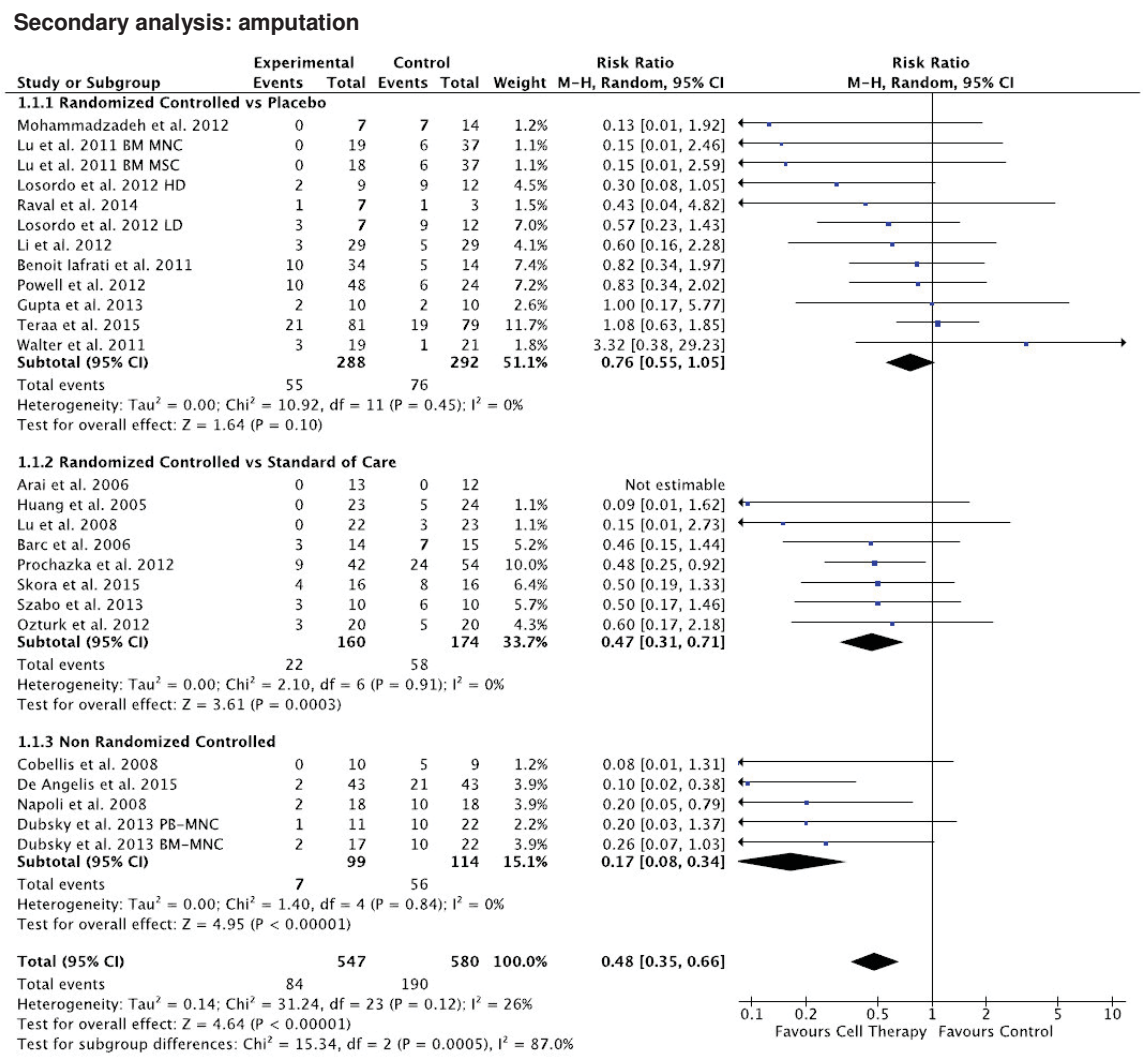


(Egger's test p = 0.86)

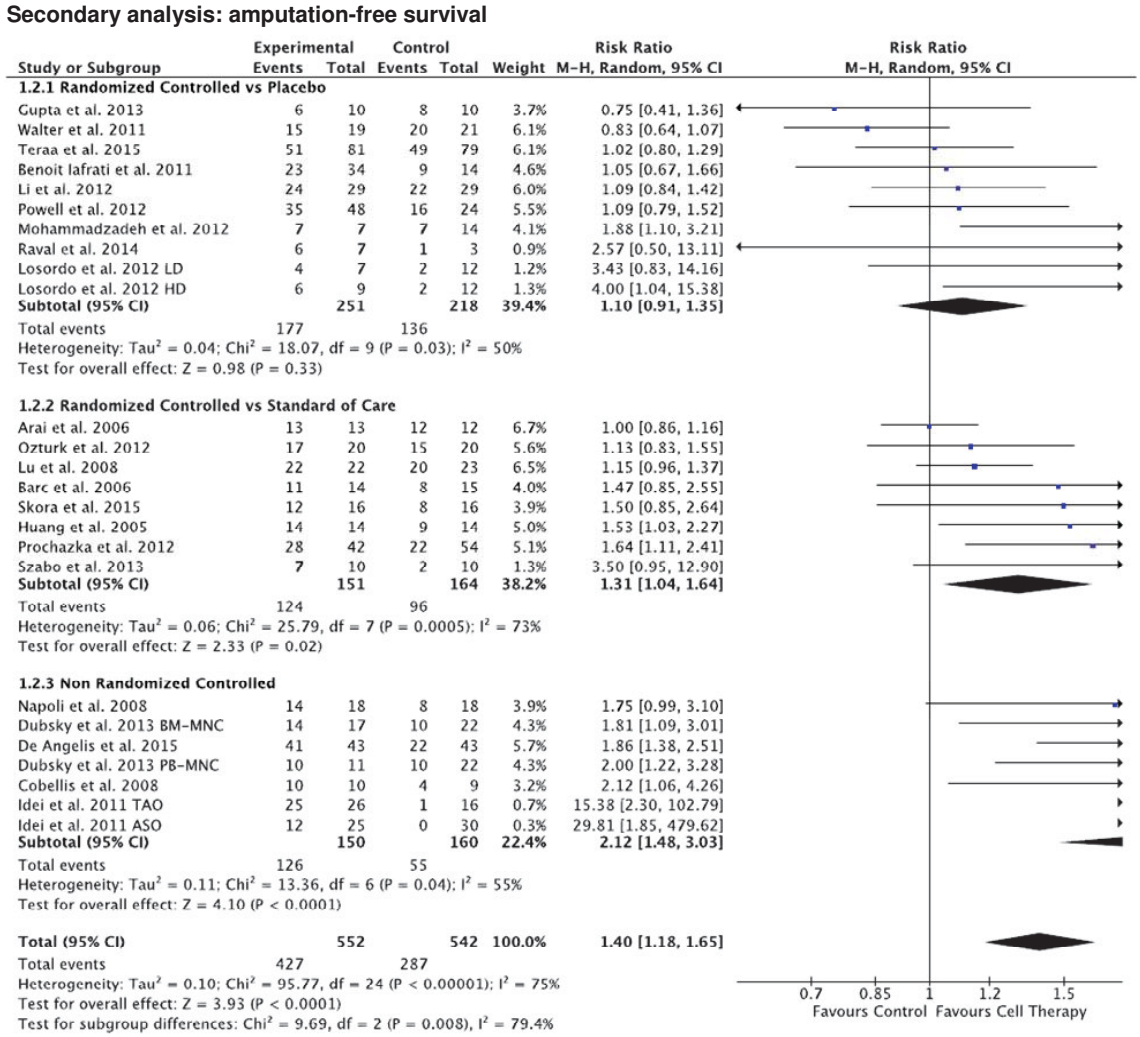


(Egger's test p = 0.23)

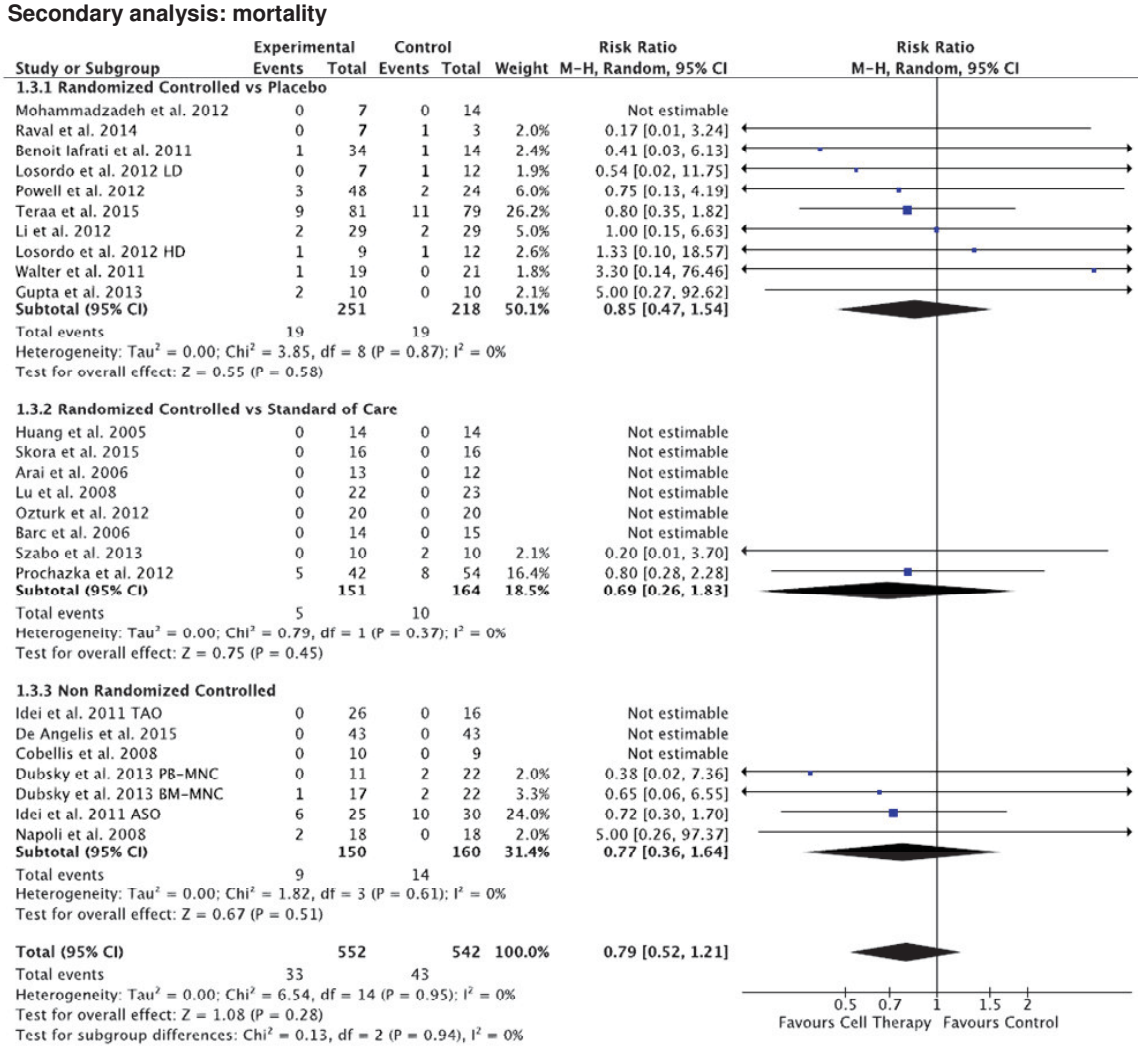
Supplemental Figure IIIA. Secondary analysis, including all controlled trials. Panel A. Amputation: the risk ratios and 95% C.I. are shown on the x-axis.



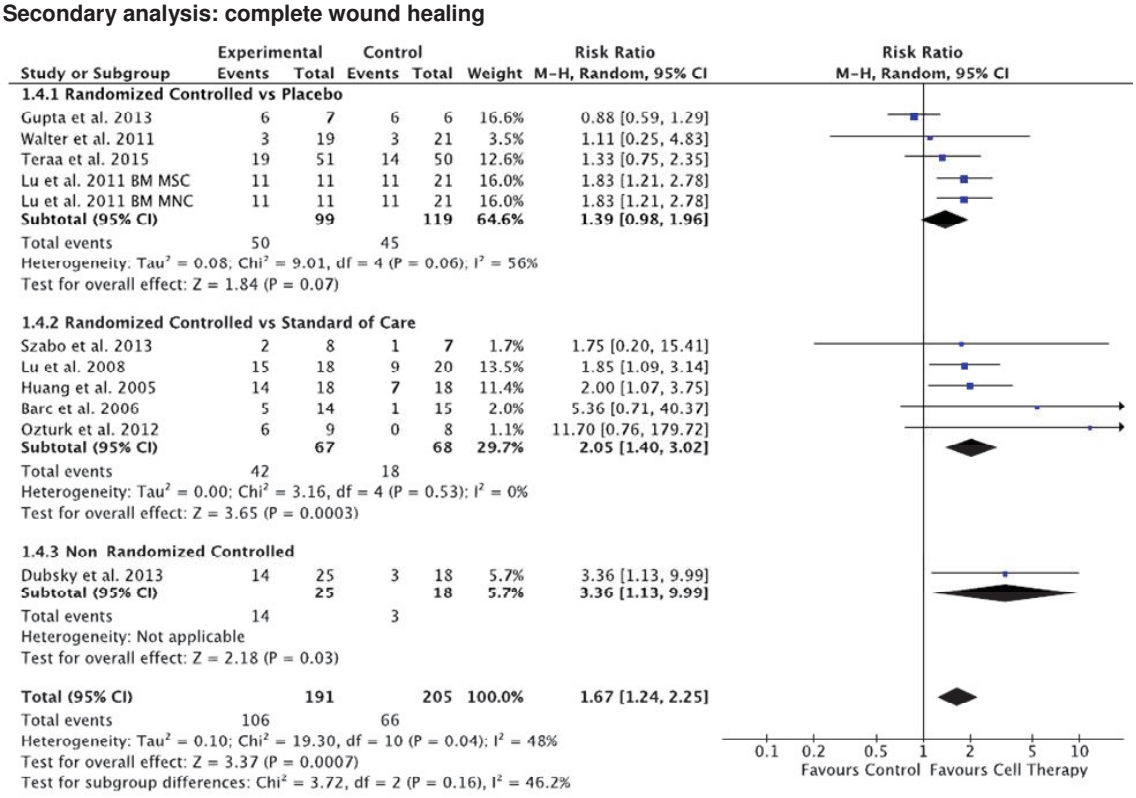
Supplemental Figure IIIB. Secondary analysis, including all controlled trials. Panel B. Amputation-free survival: the risk ratios and 95% C.I. are shown on the x-axis.



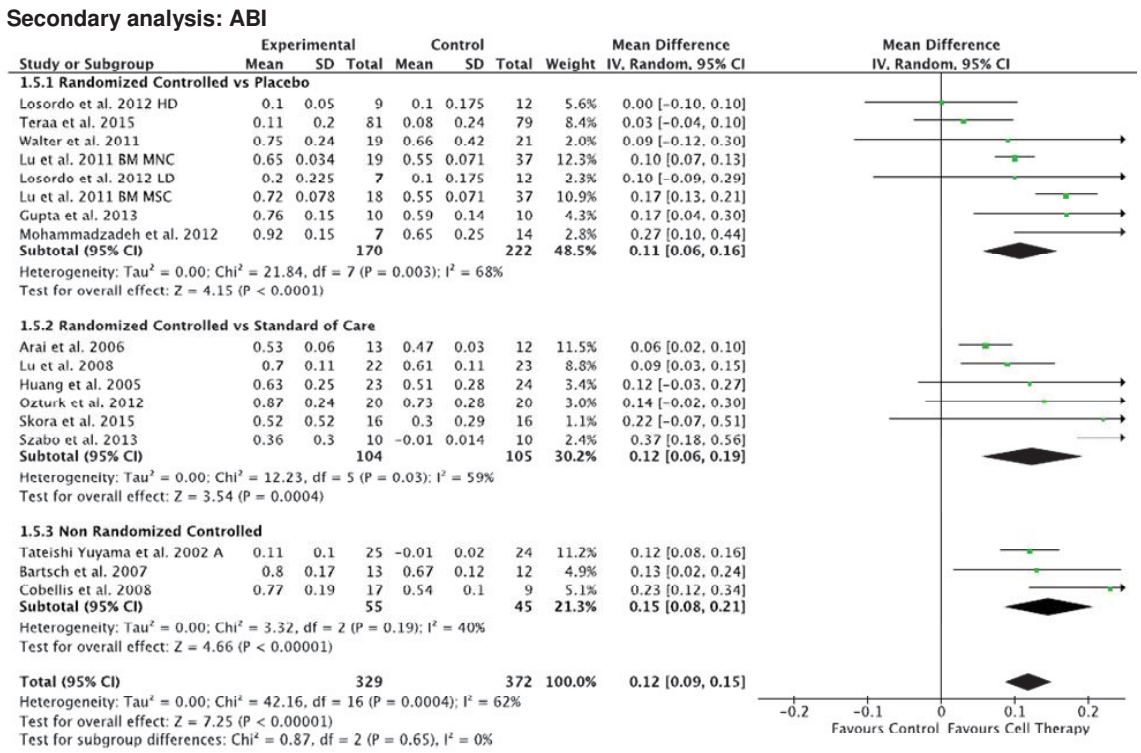
Supplemental Figure IIIC. Secondary analysis, including all controlled trials. Panel C. Death: the risk ratios and 95% C.I. are shown on the x-axis.



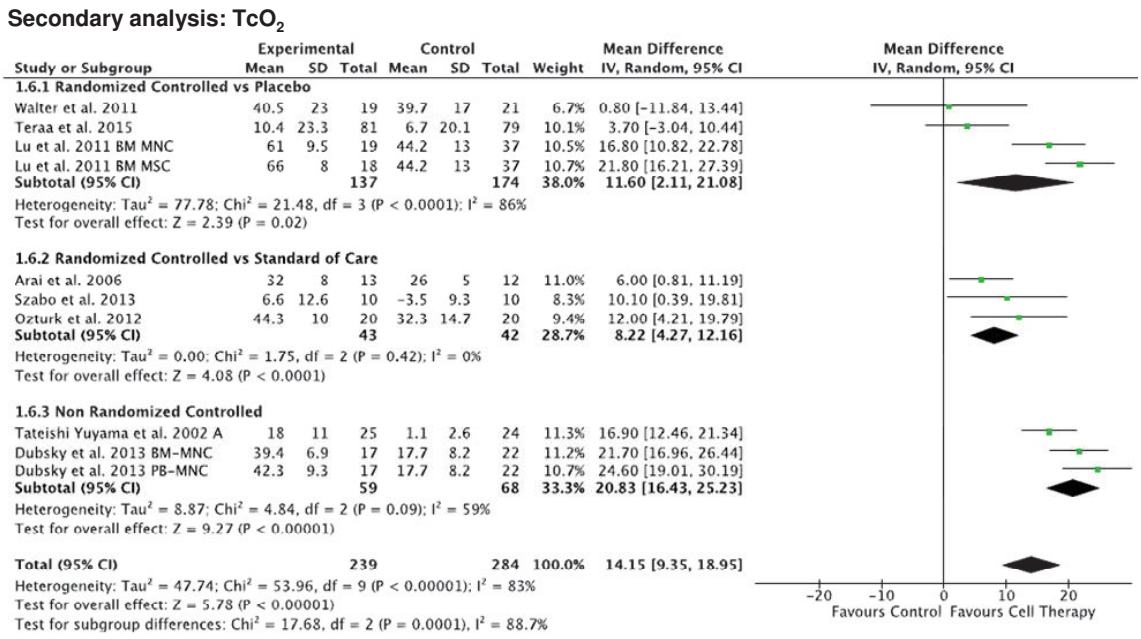
Supplemental Figure IIID. Secondary analysis, including all controlled trials. Panel D. Complete wound healing: the risk ratios and 95% C.I. are shown on the x-axis.



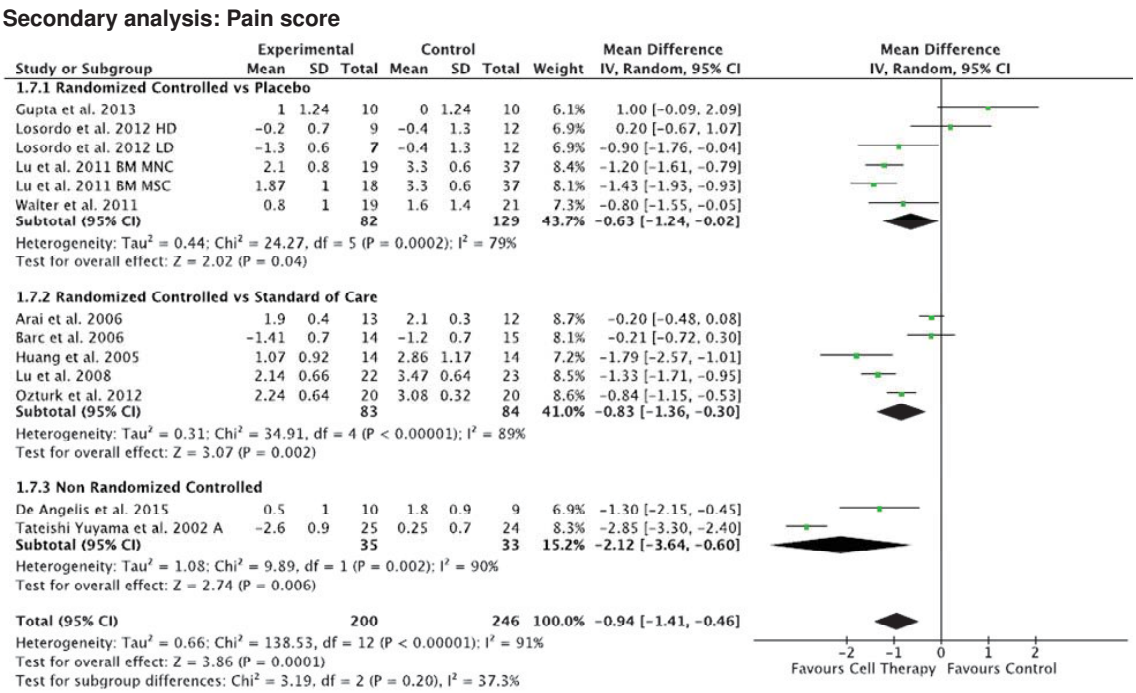
Supplemental Figure IIIE. Secondary analysis, including all controlled trials. Panel E. ABI mean differences and 95% C.I. are shown on the x-axis.



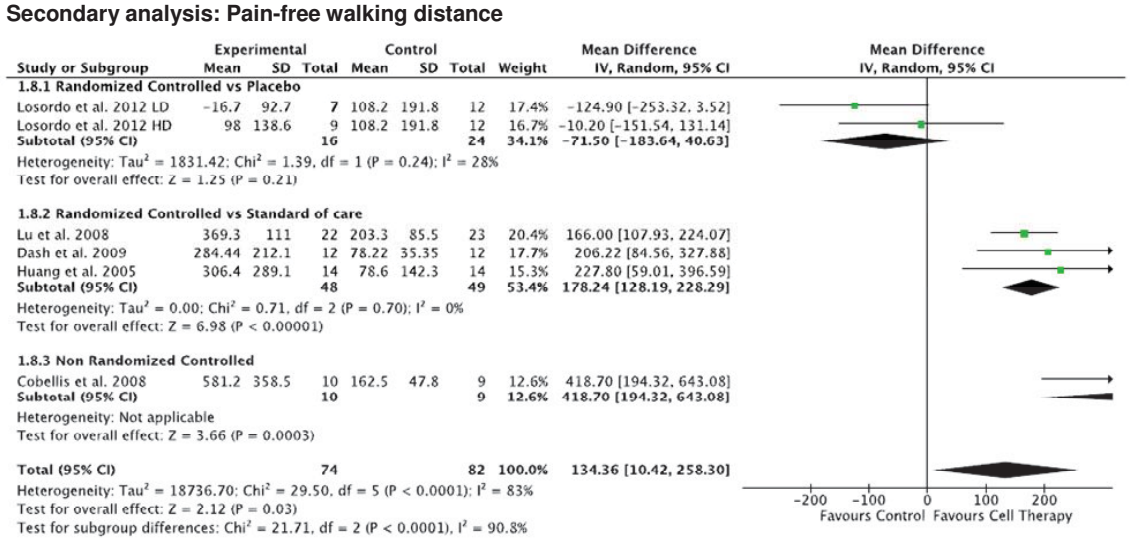
Supplemental Figure IIIF. Secondary analysis, including all controlled trials. Panel F. TcO_2 (mm Hg) mean differences and 95% C.I. are shown on the x-axis.



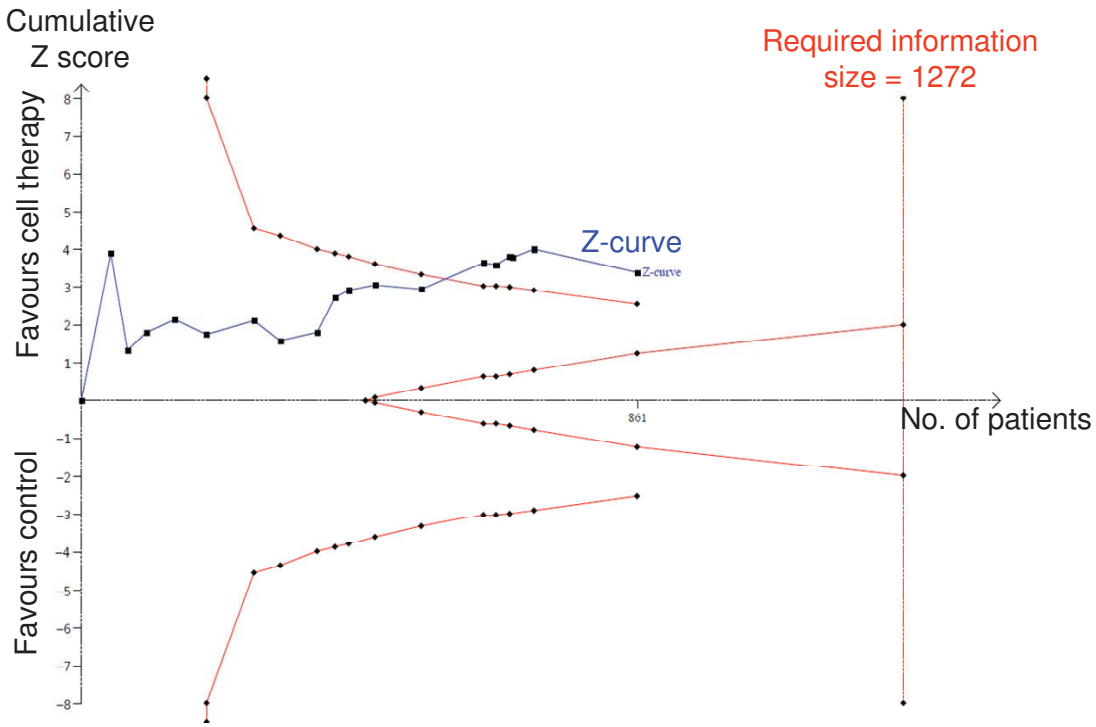
Supplemental Figure IIIG. Secondary analysis, including all controlled trials. Panel G. Rest pain score (scale 0-4) mean differences and 95% C.I. are shown on the x-axis.



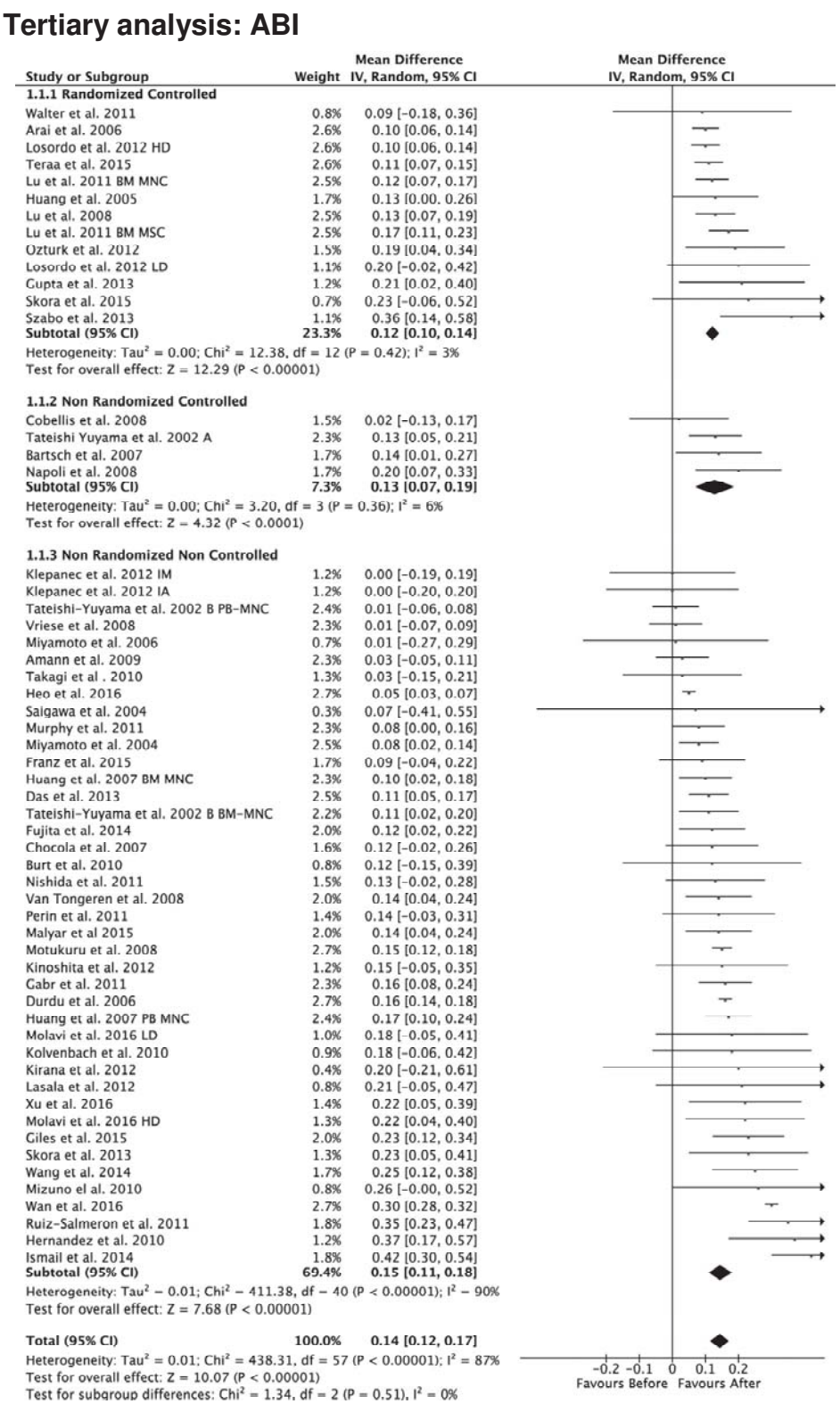
Supplemental Figure IIIH. Secondary analysis, including all controlled trials. Panel H. Pain-free walking distance (m) mean differences and 95% C.I. are shown on the x-axis.



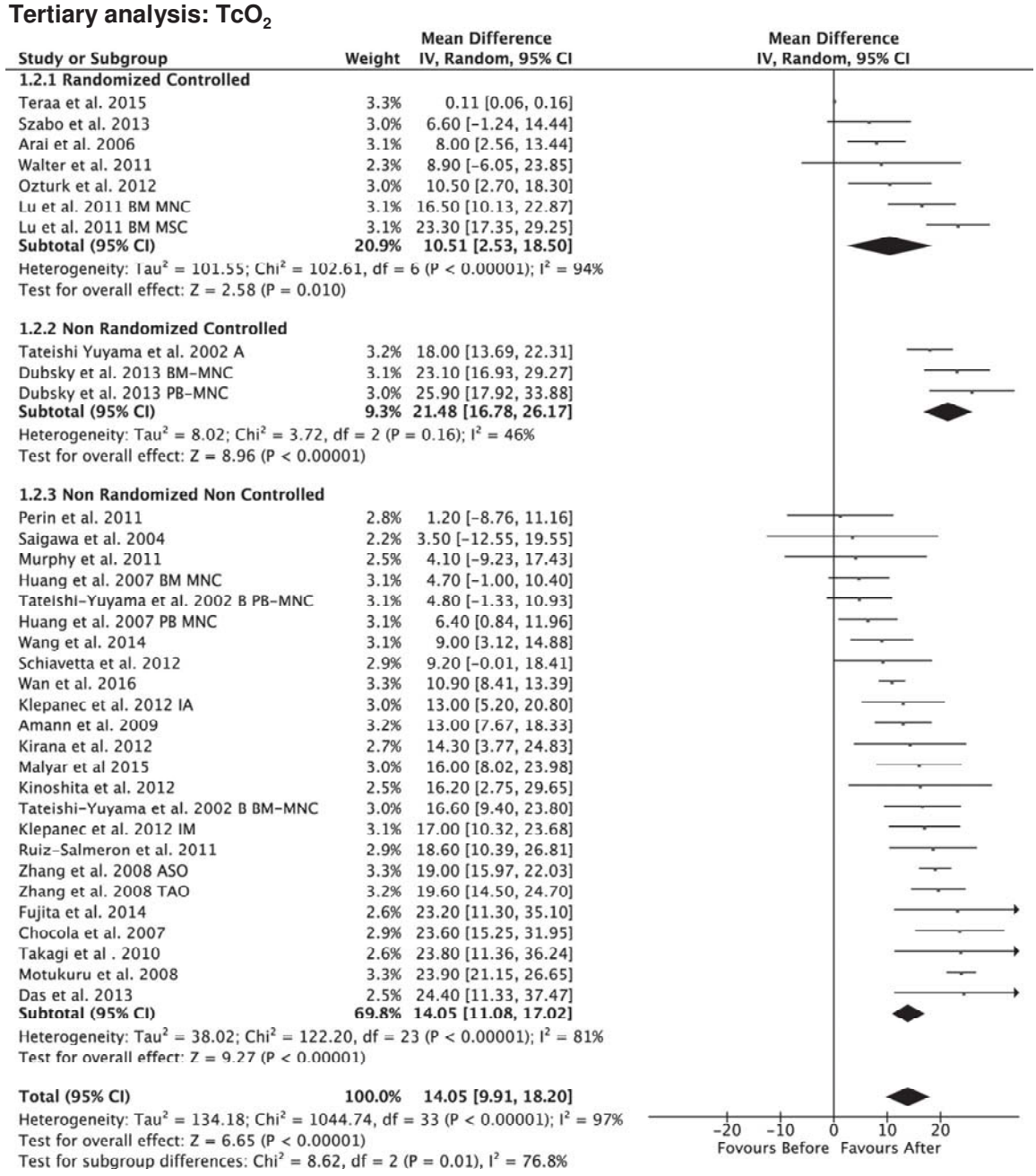
Supplemental Figure IV. Prospective trial sequential analysis of cell therapy versus control for preventing major amputation in RCTs. To the left, the red inward-sloping lines make up the trial sequential monitoring boundaries. The solid blue line is the cumulative Z curve (19 dots equal to the 19 RCTs). The graph shows that the heterogeneity-adjusted required information size to demonstrate or reject a significant effect of cell therapy on amputation (with RR calculated from placebo-controlled trials, alpha = 5%, and a beta = 10%) was 1272 patients (vertical red line).



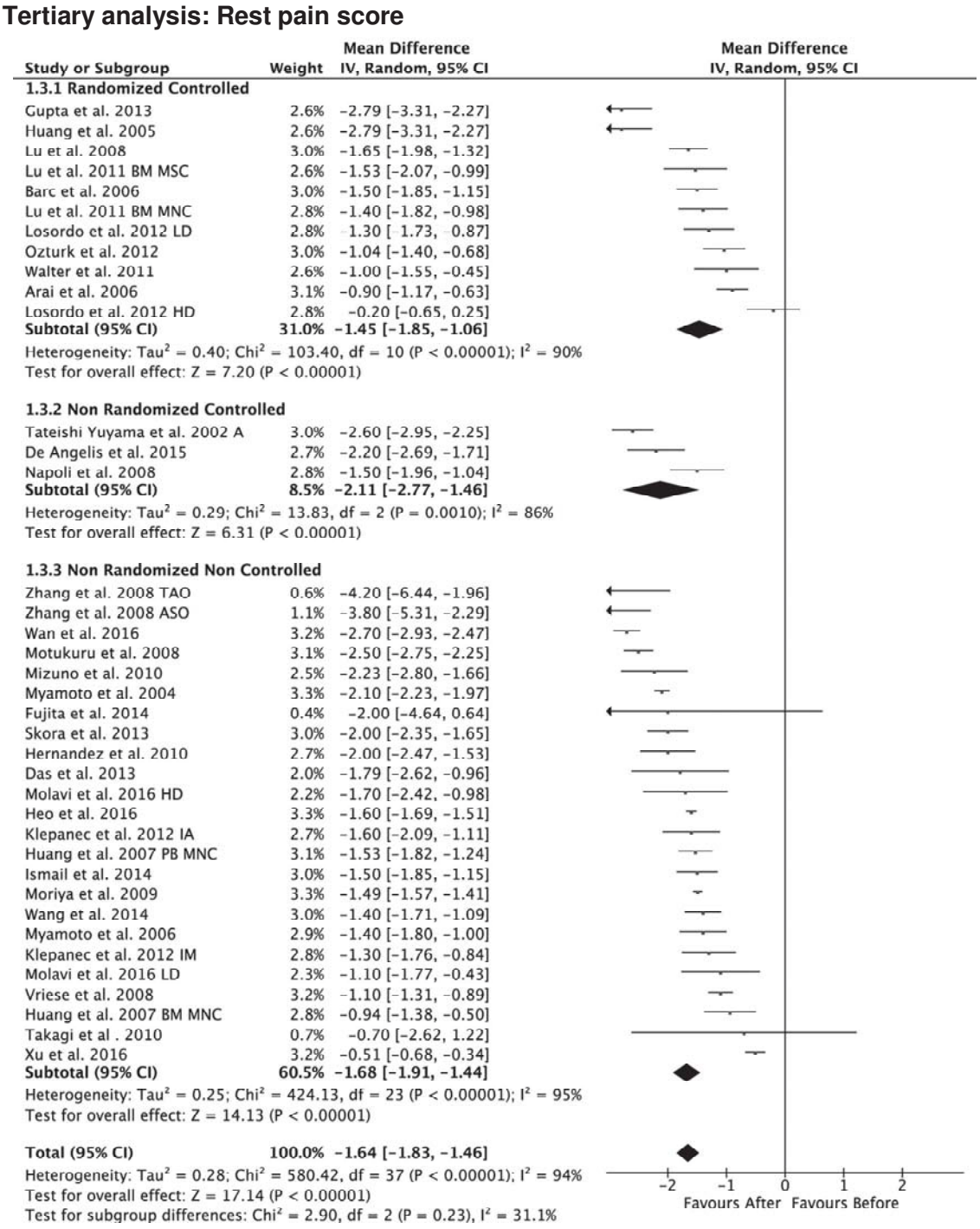
Supplemental Figure VA. Tertiary analysis, including all studies where surrogate endpoints are reported as change from baseline after cell therapy.
Panel A: Mean differences in ABI with 95% C.I. are shown on the x-axis.



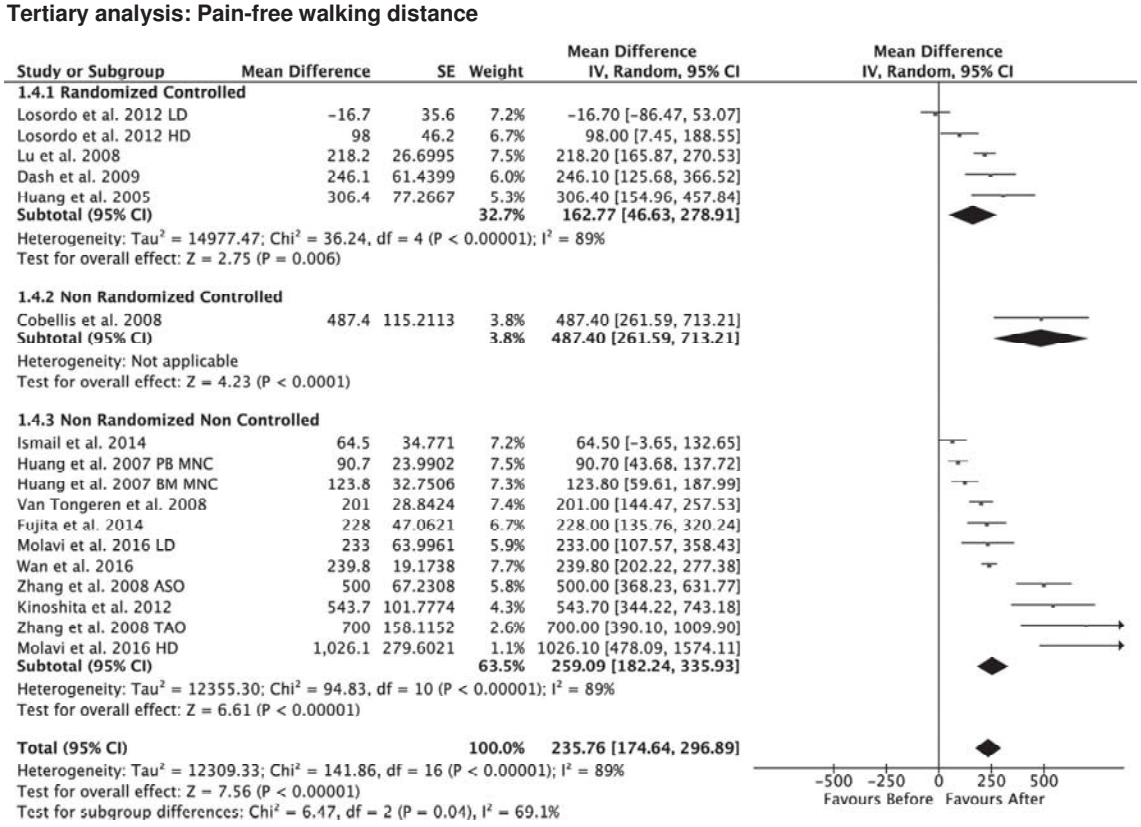
Supplemental Figure VB. Tertiary analysis, including all studies where surrogate endpoints are reported as change from baseline after cell therapy.
Panel B: Mean differences in TcO_2 (mm Hg) with 95% C.I. are shown on the x-axis.



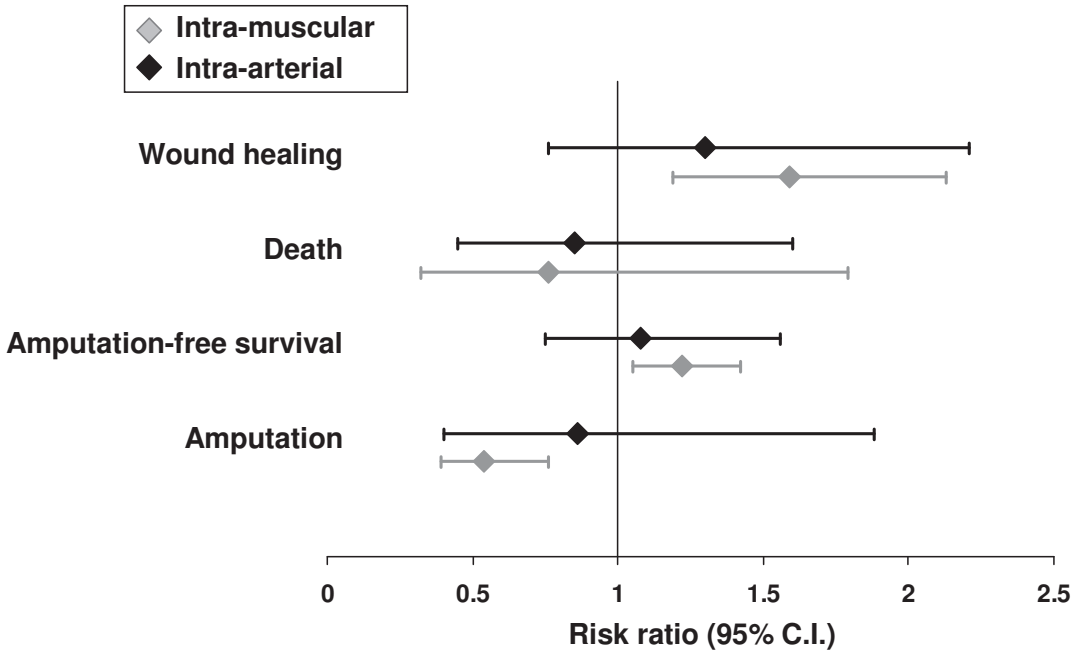
Supplemental Figure VC. Tertiary analysis, including all studies where surrogate endpoints are reported as change from baseline after cell therapy.
Panel C: Mean differences in rest pain score (scale 0-4) with 95% C.I. are shown on the x-axis.



Supplemental Figure VD. Tertiary analysis, including all studies where surrogate endpoints are reported as change from baseline after cell therapy.
Panel D: Mean differences in pain-free walking distance (m) with 95% C.I. are shown on the x-axis.



Supplemental Figure VI. Comparison intra-muscular versus intra-arterial route of cell therapy administration. See also sub-analyses reported in Table 2 of the main manuscript.



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Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5, 6

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Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5-7
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Suppl. Pag 9
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Fig 2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7, 8
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	8-10
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	11
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	11 ,12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	13

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