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Therapeutic approaches in PAH with beneficial effects on right ventricular function - preclinical studies

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NOME

André Carlos Almeida Balsa

NÚMERO DE ESTUDANTE	E-MAIL
201705745	up201705745@up.pt

DESIGNAÇÃO DA ÁREA DO PROJECTO

Ciências da saúde

TÍTULO DISSERTAÇÃO

Therapeutic approaches in PAH with beneficial effects on right ventricular function - preclinical studies

ORIENTADOR

Carmen Dulce da Silveira Brás Silva Ribeiro

COORIENTADOR

Rui Miguel da Costa Adão

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Dedicatória

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Therapeutic approaches in PAH with beneficial effects on right ventricular function - preclinical studies

André Balsa[1], Rui Adão[1,3,4], Carmen Brás-Silva[1,2*]

[1]UnIC@RISE, Department of Surgery and Physiology, Faculty of Medicine, University of Porto, Porto, Portugal

[2]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal

[3]Department of Pharmacology and Toxicology, School of Medicine, Universidad Complutense de Madrid, Madrid, Spain

[4]CIBER Enfermedades Respiratorias (Ciberes), Madrid, Spain

*** Correspondence:**
Carmen Brás Silva
carmensb@med.up.pt

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Abstract

Introduction: Pulmonary hypertension (PH) is a progressive condition that affects pulmonary vessels, but its main prognostic factor is right ventricle (RV) function. It is defined as an elevation of mean pulmonary arterial pressure above 20 mmHg at rest. PH group 1 – pulmonary arterial hypertension (PAH) – is a syndrome that primarily benefits from targeted treatment. Many mice/rat models are used for research in PAH, but results fail to translate in clinical trials. Recently, more and more studies are also using pulmonary arterial banding (PAB) – a model of RV dysfunction/failure without PH. This review aims to summarize studies that test interventions on PAB and other PH models concomitantly.

Methods: articles were searched in Scopus, Web of Science and PubMed/MEDLINE, without time or language limitation. Inclusion criteria consisted of pharmacological therapeutical interventions, tested on a PAB and in a PAH animal model. Exclusion criteria were acute interventions, genetic models, and studies without data for PAB group and, at least, one other PH model.

Results: multiple tested drugs both improved pulmonary vascular hemodynamics on PH models and ameliorated RV structure and function after PAB, in rats and mice. PH models and PAB frequently exhibited similar results (73,1% concordance) with drugs other than endothelin receptor antagonists and phosphodiesterase inhibitors. RV systolic pressure (RVSP) accounted for most differences between PH models and PAB. Only dichloroacetate improved it in PAB animals, whereas 14 out of 19 drugs/combination of drugs improved RVSP in PH models. Results on RV fibrosis, on the other hand, all agreed (12 drugs). Macitentan, sildenafil and tadalafil improved most tested pathophysiological parameters in PH models, but almost none in PAB animals: only macitentan ameliorated two – Fulton index and TAPSE. Dapagliflozin was the only drug that improved no parameters.

Conclusion: this review showed that many drugs currently under research for PAH have a cardioprotective effect on animals that may translate to humans, as well as pulmonary vascular hemodynamics and remodelling benefits. However, results of isolated studies should be interpreted with caution, as small differences in the methodology can lead to noticeable changes in the results. To improve the translational potential of drugs in this field, researchers should test them in multiple models, including PAB, while optimizing induction methods for human disease translation.

1 Introduction

Pulmonary hypertension (PH) is a progressive condition that affects pulmonary vessels, leading to the worsening of the right ventricular function(1), which is the main prognostic factor(2). It is defined as an elevation of mean pulmonary arterial pressure above 20 mmHg at rest(3). There are 5 major types of PH, based on clinical presentation, pathophysiology, and management(3). Group 1 PH is also called pulmonary arterial hypertension (PAH), and it is less common than PH groups 2 and 3(3). Most (50-60%) of PAH cases are idiopathic(3). The other most common associated conditions are connective tissue disease, congenital heart disease and portal hypertension(3). Treatment of underlying condition is possible for patients with PH group 2 (PH associated with left heart disease), group 3 (PH associated with lung diseases/hypoxia) and group 4 (PH associated with pulmonary obstructions)(3), unlike for most patients with PAH.

Many small animal models are used for research in the field of PH: from older, “classical” models – chronic hypoxia (CH) and monocrotaline (MCT) – to newer models – such as Sugen5416/hypoxia (SuHx) and pulmonary artery banding (PAB).(4) “Classical” models tend to present with a milder phenotype(4). MCT model is induced by a single injection of monocrotaline, which leads to PH, RV hypertrophy and pulmonary vascular remodelling – as in human PH – but it also affects the liver, the myocardium, and the kidney, unlike human disease(5). CH animals are exposed to a hypoxic (generally with 10% oxygen) environment for 3-4 weeks(5). This causes pulmonary vascular remodelling, which improves with normoxia(5). Therefore, they are mostly used to investigate milder forms of PH, like group 3 PH(6). SuHx animals receive an injection of a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist (semaxinib or Sugen5416) and then are exposed to hypoxia, like CH animals(5). SuHx rats have the advantage of showing pulmonary plexiform lesions – like human PAH – as well as vascular remodelling(5). PAB rats or mice undergo surgery to permanently constrict the pulmonary trunk, which leads to right ventricle remodelling, without PH(6). It is used to evaluate the direct effects of drugs on the RV(6).

Many drugs which improve PAH in small animals fail in clinical trials(7). In fact, in a recent meta-analysis, only 41 out of 522 interventions in animal models (8%) were ineffective(8). Yet only drugs targeting 3 pathways are currently approved for PAH treatment – nitric oxide, endothelin, and prostacyclin pathways – and they are all related to benefits in pulmonary vasculature(7). No approved therapy targets the RV(9). This difficulty on the translation from animal models to human has multiple explanations. Most importantly, no existing animal model replicates all features of PAH in humans(5). Some problems are milder phenotype (CH), damage in other organs (MCT) and absence of pulmonary vessels remodelling (PAB)(6). Also, depending on the model and on the methodology – type of rat/mouse, duration of induction, anaesthetic used for hemodynamic evaluation – the phenotype can greatly vary(4, 9).

The current PH animal models have similarities and differences to human PAH. Therefore, it is advantageous to use models which combine more than one hit (like SuHx), or to compare the effect of pre-clinical drugs on multiple models(10). Furthermore, as the main prognostic factor of PAH is the right ventricular function(2), direct cardioprotection – assessed by PAB – is an interesting novel option(6), so many recent papers have concomitantly evaluated potential PAH drugs in PAB plus one or more PH models. This review summarizes and analyses these studies. We aim to provide a picture of the effect of pre-clinical and clinical therapies on multiple animal models, with a special focus on PAB.

2 Methods

This review included studies from Scopus, Web of Science and PubMed/MEDLINE, without time or language limitation.

The query used was: (pulmonary hypertension OR pulmonary arterial hypertension) AND (SUGEN OR SU5416 OR (chronic hypoxia) OR monocrotaline OR MCT OR Schistosomiasis OR Schistosoma OR (Endothelin receptor-B) OR ET-B OR Angiopoeitin-1 OR Serotonin OR 5-HTT) AND (PAB OR pulmonary artery banding or PTB or pulmonary trunk banding).

2.1 Inclusion and exclusion criteria

Inclusion criteria consisted of pharmacological interventions to prevent/reverse PH, tested both on a PAB group and in a PH animal model.

Studies were excluded because of acute interventions (when the treatment was administered only once) and studies without data for PAB group and, at least, one other PH model.

2.2 Data extraction

All selected studies were carefully reviewed. We extracted data from most assessed and important outcomes related to RV. Outcomes were divided on model induction, RV structure, RV systolic function, RV diastolic function and pulmonary vascular hemodynamics and remodelling to facilitate their presentation, although some outcomes are related to more than one area.

3 Results

Nineteen studies were selected after applying exclusion and inclusion criteria (figure 1) (11-29). They were published between 2009 and 2022 and they provide results of 20 drugs and two combinations of two drugs, with a great variety of mechanisms of action (table 1). Some drugs are already in use for PAH – such as sildenafil, tadalafil and macitentan, and others are important in other diseases – like sacubitril/valsartan, dapagliflozin and ivabradine.

3.1 Methodology

Drugs were tested in 5 different PH/RV remodelling models: chronic hypoxia (CH), SU5416/hypoxia (SuHx), monocrotaline (MCT), monocrotaline + shunt (MCT+S), and pulmonary artery banding (PAB).

All MCT animals were rats (table 2). They were given a single monocrotaline dose of 60 mg/Kg, except in one group (30 mg/Kg)(14), to induce PH. In two studies, rats further underwent aortocaval shunt surgery (MCT+S)(17, 29). Other models underwent hypoxia periods: CH and SuHx (table 3). Almost all these animals were exposed to air with 10% O_2 for 3/4 weeks. In the start of the hypoxia period, SuHx rats were additionally given an VEGFR inhibitor – Sugen 5416 (semaxinib) – at a 20 or 25mg/Kg dose. Different from the other models, PAB animals underwent surgery for pulmonary artery constriction, whose grade of constriction is defined by the needle/clip size. Even for the same strains of mice/rats, the sizes greatly varied: for example, in Sprague-Dawley banded rats, the needle size ranged from 22G to 16G, and one study used a 0,9 mm diameter clip(16).

Treatment regimens also had important differences (table 2 and 3). Some studies adopted a preventive strategy, starting the treatment immediately after induction, while others waited some (maximum 4) weeks to start it – therapeutic strategy. Treatment duration ranged from 1 to 7 weeks, and its duration was the same for PH and PAB models in about half the studies.

Finally, an important variable for the final outcomes is the choice of the anaesthetic for hemodynamic measurements. In this regard, most (at least 10 in 19) of the experiments used isoflurane (table 1).

3.2 Differences of effects across different models

Overall, 63,0% of PH models results agree with the PAB results (40,3% both improve and 22,7% both have no significant effect). This percentage is bigger for structure parameters (results of table 3, plus RV weight measures, TIMP-1, and RV wall thickness) for MCT rats than SuHx animals (75,8%). The already approved therapies for PAH – macitentan, tadalafil and sildenafil – account for nearly half of the discordances (43,9%). Considering all other drugs but these 3, overall concordance raises to 73,1%, and for structure parameters in MCT rats to 92,6%.

Most used models, besides PAB, were MCT and SuHx. Concordance of MCT (65,9%) and SuHx (61,9%) with PAB results was similar.

3.3 RV structure

Results of MCT and SuHx groups were the same as PAB groups in RV fibrosis, for all the 12 drugs with data for more than one model (table 4). Sildenafil decreased TIMP-1 (marker of fibrosis) in the MCT group but had no significant effect in the PAB group (table S2) (26).

Some drugs had positive effects in PH models and PAB, some improved mostly parameters on PH models, and some improved no parameters (table 4). Urocortin-2, ivabradine, sunitinib, sorafenib, neuregulin-1 and dantrolene ameliorated multiple parameters in more than one animal model. Sildenafil, macitentan, tadalafil and the combination of the previous two had no effect on the PAB groups, except for macitentan, which ameliorated Fulton index. Sildenafil even significantly worsened PAB group cross-sectional area, despite decreasing it in the MCT group. Sacubitril/valsartan, dapagliflozin and RVX208 had no significant effect on these parameters. RVX208 further increased Fulton index in the PAB rats(29).

3.4 RV systolic function and blood pressure

RVSP and TAPSE were the most assessed RV systolic function parameters (table 5). Most of the drugs ameliorated RVSP in the PH models. However, out of 18 drugs/combination of drugs, only dichloroacetate improved RVSP of PAB animals(24). Dichloroacetate also improved pulmonary artery gradient in banded rats(24). Of all drugs with data for both parameters (16 drugs), only dapagliflozin had no significant effect on neither RVSP nor TAPSE(19). Like RV structure results, sildenafil, macitentan, tadalafil and the combination of the previous two improved no RV systolic function parameters in PAB animals, except macitentan, which ameliorated TAPSE(20).

Some studies also measured RVEF, RV fractional area change, CO, CI, MAP and SBP (table 5, S1 and S2). Urocortin-2 (MCT and PAB), GS-444217 (MCT) and neuregulin-1 (MCT) improved RV ejection fraction. Sacubitril/valsartan did not improve RVEF. Other studies lacked data. Ivabradine (SuHx and PAB), celastrol (SuHx and PAB) and gapmeR H19 (MCT and PAB) improved RV fractional area change, another measure of RV function. Gallein improved CO only on the MCT group, and CI only in the PAB group(25). Sacubitril/valsartan decreased mean arterial pressure(12). Sorafenib increased it in the MCT group(16).

3.5 RV diastolic function and pulmonary vascular remodelling

Most (14 out of 19) studies measured RVEDD and/or RVEDP (table 6). In two studies, PH induction with MCT injection did not increase RVEDD and RVEDP(14, 15). RV remodelling induction by PAB did not increase RVEDP in one study(11). A few (4) studies measured RV tau. In all these (7) groups, RV tau improved with the intervention. As a highlight, GapmeR H19 improved RVEDD and RVEDP in both MCT and PAB groups(22). Urocortin-2 and neuregulin ameliorated RVEDD, RVEDP and tau in MCT rats(11, 21). They also improved RV end-diastolic volume and stiffness (data not shown).

Most studies also provided information on pulmonary vascular remodelling (table 7). Data from total, medial and intimal thickness resulted from analysis of many different arteriole size ranges. Results were very positive: 12 out of 14 drugs improved PVR, TPR or PAAT: dapagliflozin and GapmeR H19 had no significant effect. These drugs also did not decrease mean medial thickness. Also, macitentan and tadalafil alone or combined improved TPR – but only when combined significantly decreased arteriole muscularization(20).

4 Discussion

We found that multiple drugs both improved pulmonary vascular hemodynamics on PH models and ameliorated RV structure and function after PAB, in rats and mice. With drugs other than ERA and PDE5i, PH models and PAB frequently exhibited similar results (73,1% concordance), particularly in the case of MCT rats for structure-related parameters (92,6%).

RVSP accounted for most differences between PH models and PAB. Only dichloroacetate improved it in banded animals, whereas 14 out of 19 drugs/combination of drugs improved RVSP in PH models. Results on RV fibrosis, on the other hand, all agreed (12 drugs). ERA and PDE5i – macitentan, sildenafil and tadalafil – improved most parameters in PH models, but almost none in PAB animals: only macitentan ameliorated two – Fulton index and TAPSE. Combination of macitentan and tadalafil improved pulmonary remodelling (arteriole muscularization), unlike both drugs on monotherapy. Unexpectedly, dapagliflozin was the only drug that improved no parameters.

4.1 Recent studies show multiple drugs with cardioprotective potential

PAB is a model used in PH research. Rats or mice undergo surgery to mechanically constrict the pulmonary trunk, developing RV dysfunction, without affecting pulmonary vessels(30). Therefore, if a drug improves RV function of a PAB animal, that drug likely has a direct cardioprotective action(6). In PH models, an improvement of RV function can also result of indirect action, through afterload reduction due to pulmonary effects, therefore the PAB model is useful to distinguish these effects(6).

As RV function is the main determinant of prognosis in PH(2), cardioprotection is seen as a key to improve PH treatment(6). Therefore, researchers search more and more for drugs with direct benefits on the RV. As the studies in this review are recent (more than half were published after 2018) and they use the PAB model, it is comprehensible that most drugs seem to directly protect the RV. Some documented cardioprotective mechanisms include pathways related to fibrosis (GS-444217, sorafenib, sunitinib), mitochondrial dysfunction (dichloroacetate), oxidative stress (MitoQ), and epigenetic alterations (GapmeR H19, RVW208, sodium valproate)(31).

4.2 RVSP accounted for most discordances, RV fibrosis for most concordances

In the absence of RV outflow obstruction, RVSP estimates pulmonary artery systolic pressure, which can be used to calculate mean pulmonary artery pressure (mPAP)(32). PH models, like in PH in humans, present with an elevated mPAP(4). This explains why, in all studies included in this review, PH models also exhibit an increased RVSP. Treatment with most drugs decreased RVSP, so these drugs also ameliorated mPAP.

In PAB model, there is an obstruction to RV outflow, leading to RV pressure overload(6), which causes RVSP elevation. All drugs but one had no effect on RVSP. Only dichloroacetate decreased RVSP in the PAB model(24). Dichloroacetate also reduced the pulmonary artery pressure gradient(24). This suggests that RVSP decrease was caused by reduction of pressure gradient. As PAB uses a fixed constriction on the pulmonary artery, pressure gradient should also be constant. This finding requires further research.

PH and PAB models exhibited similar RV fibrosis improvements, which suggests that these models may share common fibrotic pathways. Furthermore, 92,6% of MCT results agreed with PAB for the structure parameters, excluding ERA and PDE5i. This disagrees with a recent review on RV fibrosis due to PH which points some differences in fibrosis location and mechanisms(33). However, the review also states that RV fibrosis is an area that needs more research(33).

4.3 Results from individual studies should be interpreted with caution

Other studies tested these drugs on animal models and results lacked consistency. In Schafer et al study(26), sildenafil treatment for 3 weeks, immediately after PAB surgery in rats, had no effect on the RV function and structure, except increasing the cardiomyocyte cross-sectional area. Rai et al(34) found similar results after 4 weeks of treatment. Borgdorff et al(35, 36) obtained different results, depending on the treatment regimen. Preventive strategy, with 4 weeks of sildenafil from PAB surgery day 1, resulted in RV systolic function improvement, no effect on RV diastolic function, and RV fibrosis worsening(35). Therapeutical strategy, based on sildenafil treatment starting 4 weeks after surgery, for 4 weeks, resulted in RV systolic and diastolic improvement, and RV fibrosis reduction(36). Studies on MCT rats showed improvements on RV systolic and diastolic function, and structure(37-39).

Sildenafil, as tadalafil, is a PDE5 inhibitor. PDE5 is an abundant enzyme in the lung vasculature that degrades cGMP(40). Its inhibition leads to vasodilation, improving pulmonary hemodynamics(40). There is also evidence of direct cardioprotective effects(41). However, these effects may be of a lesser importance in PAH, as studies with PH models show benefits, but many with PAB reveal absence of improvements, of even worsening of RV fibrosis.

Unlike Li et al(19), in posterior studies dapagliflozin improved RV function, RV hypertrophy, and pulmonary vascular remodelling in MCT rats(42, 43). Tang et al attributed the difference to the lower mortality of MCT rats and to the longer duration of treatment: 3 weeks instead of 2 weeks(42). Wu et al treated rats for even longer: 5 weeks from MCT injection(43).

Andersen et al reported that sacubitril/valsartan improved some parameters only in SuHx rats and mean arterial pressure also in PAB(12). In other studies, sacubitril/valsartan also improved Fulton index, RV wall thickness, fibrosis and RVEDP, in MCT and SuHx animals(44, 45). A study found a RVSP and RV hypertrophy reduction in PAB animals(46).

As the study included in this review(14) – which tested sodium valproate in MCT and PAB rats – other studies showed beneficial effects of valproic acid on CH and MCT plus CH animals(47, 48). A study reported multiple detrimental effects of trichostatin A(49), another histone deacetylase inhibitor, in PAB rats: it worsened fibrosis, RV dilation, cardiac output, TAPSE, and more parameters.

To sum up, these different findings can be related to the methodology, particularly in the induction of the models. The most important factor for hemodynamic, structural and vascular worsening is the induction period, the longer, the more severe the phenotype(4). Additionally, older models – CH and MCT – cause milder phenotypes, some anaesthetics influence RVSP and mPAP values (greater pressure values are obtained with isoflurane), and preventive strategies lead to better outcomes than therapeutic ones(4). In the PAB model, a tighter constriction of the pulmonary artery causes a more severe phenotype, ranging from RV adaptative dysfunction to RV failure(50).

4.4 Some drugs are already approved and other are being evaluated in clinical trials

Some of the drugs considered in this review are already approved for PAH. 2022 ESC/ERS guidelines for PH(3) recommend the use of PDE5 inhibitors and/or ERA in some patients, depending on cardiopulmonary comorbidities and mortality risk, due to many favourable effects on clinical trials. PDE5 inhibitors improve hemodynamics, functional class and 6-minute walk distance(51). They also reduce mortality(51). In the REPAIR clinical trial, macitentan (ERA) improved pulmonary hemodynamics, RV function and structure(52), like in SuHx rats (citação). AMBITION clinical trial compared combination and monotherapy of ambrisentan – another ERA – and tadalafil(53). Combination therapy further reduced morbidity and improved 6-minute distance(53).

Other drugs have been already tested in smaller clinical trials and observational studies, with positive outcomes. Dichloroacetate improved mPAP and PVR in genetically susceptible patients(54). Sacubitril/valsartan also reduced mPAP, in patients with heart failure with reduced ejection fraction (HFrEF)(55) and preserved ejection fraction (HRpEF)(56). A positive effect on RV function, assessed by TAPSE, was only present in HFrEF(55). Ivabradine led to functional improvements in 10 PAH patients with high heart rates(57). Sorafenib showed improvements in patients with refractory PAH(58). However, in other study, without placebo group, sorafenib led to a decrease in the cardiac index and a non-significant increase in systemic blood pressure(59).

4.5 Small findings can be important

In one article(25), cardiac output (in mL/min) and cardiac index (mL/min/g) were assessed. Gallein treatment improved cardiac output only in MCT rats, and cardiac index only in PAB animals: the bodyweght indexing affected the results. Other studies show significant differences between the control and MCT groups (neuregulin), MCT and MCT + treatment (neuregulin), sham and PAB (ivabradine), and PAB and PAB + treatment (ivabradine). Borderline cardiac output improvements can become significant with or without indexing.

Also, unlike their monotherapy, macitentan plus tadalafil improved pulmonary vascular remodelling in MCT rats(20). This was the only advantage of the combination. Accordingly, in ABMITION clinical trial, combination of ERA and PDE5i had benefits compared to both monotherapies(53).

4.6 Limitations

One limitation of this review is the absence of statistic tests. Many studies did not present the absolute values, and considering the high heterogeneity in the methods, statistic comparisons would be hard to interpret. Also, this study does not include all drugs tested on PAB model. Although it would be interesting to have a picture of all potentially cardioprotective drugs, analysing only studies which test two or more models allows to understand, for each molecule, which seem to have direct, indirect, or mixed cardioprotective effects. Also, as the same research group performs the experiments on both models, this decreases heterogeneity. One more limitation is the absence of drugs which are known to lack benefits on PAH. They would be useful for comparison purposes. In this study, only dapagliflozin completely lacked benefits, but even this drug showed improvements in animal models and in some patients. Finally, this review does not include all results of studies: some important outcomes may have been missed and the proportion of similarities/differences between models can be unrepresentative of the full results.

5 Conclusion

This review showed that many drugs currently under research for PAH have a cardioprotective effect on animals that may translate to humans, as well as pulmonary vascular hemodynamics and remodelling benefits. However, results of isolated studies should be interpreted with caution, as small differences in the methodology can lead to noticeable changes in the results. To improve the translational potential of drugs in this field, researchers should test them in multiple models, including PAB, while optimizing induction methods for human disease translation.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

AB conceived the idea and has been involved in reviewing the literature and drafting the manuscript. RA has made substantial contributions to conception and design and has been involved in drafting the manuscript. CB-S has made substantial contributions to conception and design and has been involved in revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Tables and figures

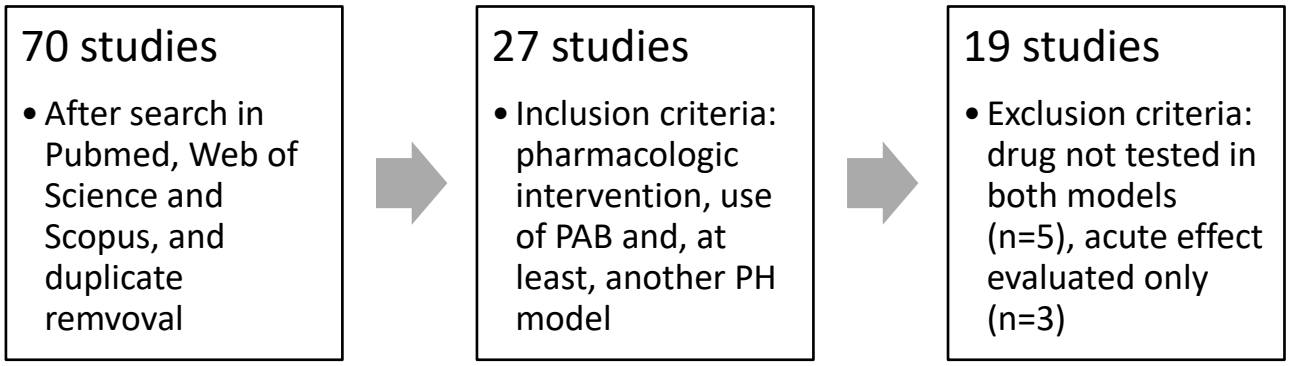

Figure 1 – study selection

571 Table 1 – Selected articles and characteristics

Study	Author	Year	PH models	Drug(s) tested	Anesthesic	Mechanism of action
1	Adão, R. et al.	2018	PAB, MCT	Urocortin-2	Sevoflurane	Type 2 CRH receptor activator
2	Andersen, S. et al.	2019	PAB, SuHx	Sacubitril/valsartan	Sevoflurane	Angiotensin II receptor/neprilysin inhibitors
3	Budas, G. R. et al.	2018	PAB, MCT, SuHx	GS-444217	Isoflurane (MCT and PAB), xylazine+ketamine (SuHx)	ASK1 inhibitor
4	Cho, Y. K. et al.	2010	PAB, MCT	Sodium valproate	(no haemodynamic evaluation)	Histone deacetylase inhibitor
5	Ishii, R. et al.	2020	PAB, MCT, SuHx	Ivabradine	Isoflurane	I_f current inhibitor
6	Kojonazarov, B. et al.	2013	PAB, MCT	Sunitinib, sorafenib	Isoflurane	PDGFR-, VEGFR- and KIT-inhibitor; raf1/b-, VEGFR-, PDGFR-inhibitor
7	Kurakula, K. et al.	2022	PAB, MCT + shunt	Juglone	ND	Pin1 inhibitor
8	Kurosawa, R. et al.	2021	PAB, SuHx	Celastrol	Isoflurane	Bsg, CyPA and NF-Kb inhibitor
9	Li, H. et al.	2021	PAB, MCT	Dapagliflozin	Pentorbital	SGTL-2 inhibitor
10	Mamazhakypov, A. et al.	2020	PAB, SuHx	Macitentan, tadalafil	Isoflurane	Endothelin-1 receptor antagonist; PDE5 inhibitor
11	Mendes-Ferreira, P. et al.	2016	PAB, MCT	Neuregulin-1	Sevoflurane, fentanyl, midazolam	ErbB family tyrosine kinase receptors activator
12	Omura, J. et al.	2020	PAB, MCT	GapmeR H19	Isoflurane	lncRNA H19 suppressor
13	Pak, O. et al.	2018	PAB, CH	MitoQ	ND	Mitochondria-targeted antioxidant
14	Piao, L. et al.	2010	PAB, MCT	Dichloroacetate	Isoflurane	Pyruvate dehydrogenase kinase inhibitor
15	Piao, L. et al.	2012	PAB, MCT	Gallein	Isoflurane	Gβγ–GRK2 signaling inhibitor
16	Schafer, S. et al.	2009	PAB, MCT	Sildenafil	Pentorbital, isoflurane	PDE5 inhibitor
17	Sun, X. Q. et al.	2021	PAB, SuHx	Clorgyline	Isoflurane	MAO-A inhibitor
18	Tanaka, S. et al.	2022	PAB, MCT	Dantrolene	Medetomidin, midazolam, butorphanol	Cardiac ryanodine receptor (RyR2) stabilizer
19	Van der Feen, D. E. et al.	2019	PAB, MCT + shunt, SuHx	RVX208	ND	BET inhibitor (BRD4 antagonist)

572 Anaesthetic: anaesthetic used for hemodynamical evaluation; CH: chronic hypoxia; MCT: monocrotaline; ND: no data; PAB: pulmonary artery banding;
573 PH: pulmonary hypertension; SuHx: Sugen 5416/hypoxia

Table 2 – Methods overview of studies using MCT or MCT + shunt models

Study	Intervention (drug)	Model MCT	Model PAB	Sex MCT	Sex PAB	MCT dose (mg/Kg)	PAB needle/clip size	Induction to intervention period MCT	Induction to intervention period PAB	Intervention period MCT	Intervention period PAB
1	Urocortin-2	WR	=	Male	=	60	16G	2 weeks	=	10 days	=
3	GS-444217	SDR	CM	Male	=	60	Clip 0,3mm	1 week	=	3 weeks	2 weeks
4	Sodium valproate	SDR	=	Male	=	30	22G	0 days	0 days	3 weeks	=
5	Ivabradine	SDR	=	Both	=	60	18G	2 weeks	=	3 weeks	=
6	Sunitinib/ Sorafenib	SDR	=	Both	=	60	Clip 0,9mm	3 weeks	2 weeks	2 weeks	=
7	Juglone (shunt)	WR	=	Male	=	60	18G	3 weeks	4 weeks	2 weeks	4 weeks
9	Dapagliflozin	SDR	=	Male	=	60	18G	2 weeks	=	3 weeks	2 weeks
11	Neuregulin-1	WR	=	Male	=	60	16G	2 weeks	=	1 week	=
12	GapmeR H19	SDR	=	Male	=	60	19G	2 weeks	3 weeks	2 weeks	5 weeks
14	Dichloroacetate	SDR	=	Male	=	60	16G	10 days	0 days	3 weeks	7 weeks
15	Gallein	SDR	=	Both	=	60	18G	2 weeks	=	2 weeks	=
16	Sildenafil	SDR	WR	Male	=	60	18G	2 weeks	0 days	2 weeks	3 weeks
18	Dantrolene	SDR	=	Male	=	60	18G	0 days	-1 week	4 weeks	=
19	RVX208 (shunt)	WR	=	Male	=	60	ND	3 weeks	4 weeks	2 weeks	4 weeks

=: same as MCT values; CM: C57BL/6 mice; MCT: monocrotaline; ND: no data; PAB: pulmonary artery banding; SDR: Sprague/Dawley rats; SuHx: Sugen 5416/hypoxia; WR: wistar rats

Induction to intervention period MCT: time from monocrotline injection until the start of the treatment

Induction to invervention period PAB: time from PAB surgery to the start of the treatment (in one study, treatment started before surgery, so this value is negative)

Table 3 – Methods overview of studies using CH or SuHx models

Study	Intervention (model)	Model CH/ SuHx	Model PAB	Sex CH/ SuHx	Sex PAB	SU5416 dose (mg/Kg)	Hipoxia time (weeks)	PAB needle/ clip size	Induction to intervention period CH/SuHx	Induction to intervention period PAB	Intervention period CH/SuHx	Intervention period PAB
2	Sacubitril/valsartan (SuHx)	SDR	WR	Male	=	25	4	Clip 0,7mm	2 weeks	=	5 weeks	=
3	GS-444217 (SuHx)	SDR	CM	Male	=	ND	4	Clip 0,3mm	-4 weeks	1 week	4 weeks	2 weeks
5	Ivabradine (SuHx)	SDR	=	Both	=	20	3	18G	0 weeks	2 weeks	3 weeks	=
8	Celastrol (CH/SuHx)	SDR	CM	Male	=	-/20	4/3	25G	-4/0 weeks	0 weeks	4/2 weeks	3 weeks
10	Macitentan/Tadalafil/ Macitentan+Tadalafil (SuHx)	WKR	=	Male	=	20	3	18G	2 weeks	1 week	2 weeks	=
13	MitoQ (CH)	CM	=	Both	=	-	4	Clip 0,35mm	-4 weeks	0 weeks	4 weeks	=
17	Clorgyline (SuHx)	SDR	WR	Male	=	25	4	Clip 0,6mm	4 weeks	2 weeks	3 weeks	6 weeks
19	RVX208 (SuHx)	SDR	WR	Male	=	20	3	ND	3 weeks	4 weeks	4 weeks	=

=: same as CH/SuHx values; CH: chronic hypoxia; CM: C57BL/6 mice; ND: no data; PAB: pulmonary artery banding; SDR: Sprague/Dawley rats; SuHx: Sugen 5416/hypoxia; WKR: wistar/kyoto rats; WR: wistar rats

Induction to invervention period CH/SuHx: time from the end of the hypoxia period to the start of the treatment (some studies start the intervention during the hypoxia period; in such cases this value is negative)

Induction to invervention period PAB: time from PAB surgery to the start of the treatment

Table 4 – Results of studies related to RV structure

		Fulton index				RV Fibrosis				CSA/D				BNP/NT-proBNP			
	Drug(s)	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P
1	Urocortin-2		↓		↓		↓		↓		↓		↓		↓		
2	Sacubitril/valsartan			↔	↔			↔	↔							↔	↔
3	GS-444217		↓	↓	↓				↓				↔		↓	↓	
4	Sodium valproate		↓		↓												
5	Ivabradine						↓	↓	↓		↓	↓	↓				
6	Sunitinib		↓		↓		↓		↓				↓		↓		↓
6	Sorafenib		↓		↓		↓		↓				↓		↓		↓
7	Juglone		↔		↓				↔								
8	Celastrol	↓		↓				↓	↓				↓				
9	Dapagliflozin			↔	↔	↔			↔								
10	Macitentan			↓	↓			↔	↔							↓#	↔#
10	Tadalafil			↓	↔			↔	↔							↓#	↔#
10	Mac. + Tad.			↓	↔			↔	↔							↓#	↔#
11	Neuregulin-1		↓		↓		↓		↓		↓		↓		↓		
12	GapmeR H19		↔		↔		↓		↓		↓		↓				
13	MitoQ	↓			↓												
14	Dichloroacetate		↓														
15	Gallein		↔		↔												
16	Sildenafil		↓		↔						↓		↑		↓		↔
17	Clorgyline			↓	↔			↔	↔*			↓	↔				
18	Dantrolene						↓				↓		↓				
19	RVX208		↔	↔	↑				↔				↔				

CSA/D: cardiomyocyte cross-sectional area/diameter; C: chronic hypoxia; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; P: pulmonary artery banding

↓ - significant decrease in the parameter

↑ - significant increase in the parameter

↔ - no significant effect in the parameter

* - in the animal model, the parameter did not significantly worsen

- NT-proBNP

610 Table 5 – Results of studies related to RV systolic function and MAP

		RVSP				TAPSE				CO				CI				MAP		
Study	Drug(s)	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P
1	Urocortin-2		↓		↔		↑				↑		↔*								
2	Sacubitril/valsartan			↓	↔			↔	↔							↔	↔			↓	↓
3	GS-444217				↔				↑		↑		↑						↔	↔	↔
4	Sodium valproate																				
5	Ivabradine		↔	↔	↔		↔	↑	↑		↑	↑	↑								
6	Sunitinib		↓		↔		↑		↑						↑		↑		↔		↔
6	Sorafenib		↓		↔		↑		↑						↑		↔		↑		↔
7	Juglone								↔								↔				
8	Celastrol	↓		↓	↔			↑	↑			↑	↑								
9	Dapaglifozine		↔		↔		↔		↔												
10	Macitentan			↓	↔			↑	↑			↑	↔							↔	↔*
10	Tadalafil			↓	↔			↑	↔			↑	↔							↔	↔*
10	Mac. + Tad.			↓	↔			↑	↔			↑	↔							↔	↔*
11	Neuregulin-1		↓								↑		↔								
12	GapmeR H19		↔		↔		↑		↑		↑		↑								
13	MitoQ	↔			↔	↔			↑	↔			↔								
14	Dichloroacetate		↓		↓						↑		↑								
15	Gallein		↔		↔		↑				↑		↔		↔		↑				
16	Sildenafil		↓		↔										↑		↔		↔		↔
17	Clorgyline			↓	↔			↔	↔			↔									
18	Dantrolene		↓		↔						↑										
19	RVX208			↓					↔			↔	↔								

611 CI: cardiac index; CO: cardiac output; MAP: mean arterial pressure RVSP: right-ventricular systolic pressure; TAPSE: tricuspid annular plane systolic
612 excursion; C: chronic hypoxia; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; P: pulmonary artery banding
613 ↓ - significant decrease in the parameter
614 ↑ - significant increase in the parameter
615 ↔ - no significant effect in the parameter
616 * - in the animal model, the parameter did not significantly worsen

617

Table 6 – Results of studies related to RV diastolic function

		RVEDD				RVEDP				Tau			
Article	Drug(s)	C	M	S	P	C	M	S	P	C	M	S	P
1	Urocortin-2		↓		↔		↓		↔*		↓		
2	Sacubitril/valsartan							↔	↔				
3	GS-444217				↓								
4	Sodium valproate		↔*		↓								
5	Ivabradine		↔	↓	↔		↔*	↓	↓		↓	↓	↓
6	Sunitinib		↓		↓								
6	Sorafenib		↓		↓								
7	Juglone												
8	Celastrol			↓					↓				
9	Dapagliflozin												
10	Macitentan			↓	↓								
10	Tadalafil			↓	↔								
10	Mac. + Tad.			↓	↔								
11	Neuregulin-1		↓				↓				↓		
12	GapmeR H19		↓		↓		↓		↓				
13	MitoQ	↓			↓								
14	Dichloroacetate												
15	Gallein												
16	Sildenafil						↓		↔				
17	Clorgyline			↔	↔								
18	Dantrolene						↔		↓		↓		↓
19	RVX208												

RVEDD: right-ventricular end-diastolic diameter; RVEDP: right-ventricular end-diastolic pressure; Tau: right-ventricular relaxation time constant; C: chronic hypoxia; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; P: pulmonary artery banding

↓ - significant decrease in the parameter

↑ - significant increase in the parameter

↔ - no significant effect in the parameter

* - in the animal model, the parameter did not significantly worsen

Table 7 – Results of studies related to pulmonary vascular hemodynamics and remodelling

		PVR			PAAT			Complete muscularization			Medial/wall thickness			TPR		
Study	Drug(s)	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S
1	Urocortin-2		↓									↓[1]				
2	Sacubitril/valsartan												↓[2]			
3	GS-444217		↓							↓						
4	Sodium valproate															
5	Ivabradine															
6	Sunitinib					↑			↓						↓	
6	Sorafenib					↑			↓						↓	
7	Juglone											↔[1]				
8	Celastrol						↑						↓[3]			
9	Dapagliflozin					↔						↔[4]				
10	Macitentan									↔						↓
10	Tadalafil									↔						↓
10	Mac. + Tad.									↓						↓
11	Neuregulin-1		↓			↑						↓[4]				
12	GapmeR H19							↔				↔[5]			↔	
13	MitoQ															
14	Dichloroacetate					↑										
15	Gallein															
16	Sildenafil															
17	Clorgyline												↔[6]			↓
18	Dantrolene											↓[7]				
19	RVX208		↔	↓								↔[1]	↔[1]			

PAAT: pulmonary artery acceleration time; PVR: pulmonary vascular resistance; TPR: total pulmonary resistance; C: chronic hypoxia; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; [1] medial thickness of small vessels (<50 µm); [2] reduction in wall thickness in arterioles of 30-60 µm, but not <30 µm and >60 µm; [3] medial thickness of distal pulmonary arteries (50-100 µm); [4] pulmonary arterial medial wall thickness; [5] medial thickness of small vessels (<100 µm); [6]clorgyline reduced intimal thickness, but not medial thickness in pulmonary arterioles (25-100 µm); [7]medial wall thickness

↓ - significant decrease in the parameter

↑ - significant increase in the parameter

↔ - no significant effect in the parameter

* - the parameter did not significantly worsen with the model induction

Table S1 – Other results

		RVEF				mPAP				RV/BW				RV weight				RV/TL			
Study	Drug(s)	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P
1	Urocortin-2		↑		↑*																
2	Sacubitril/valsartan			↔	↔							↓	↔								
3	GS-444217		↑				↓	↓											↓		
4	Sodium valproate										↓		↓								
5	Ivabradine																				
6	Sunitinib																				
6	Sorafenib																				
7	Juglone						↓										↔				
8	Celastrol																				
9	Dapaglifozine																				
10	Macitentan																				
10	Tadalafil																				
10	Mac. + Tad.																				
11	Neuregulin-1		↑																↓		
12	GapmeR H19																				
13	MitoQ																				
14	Dichloroacetate																				
15	Gallein																				
16	Sildenafil														↓		↔				
17	Clorgyline																				
18	Dantrolene										↓										
19	RVX208						↓	↔													

mPAP: mean pulmonary arterial pressure; RV: right ventricle; RV/BW: right ventricle weight/bodyweight; RVEF: right-ventricular ejection fraction; RV/TL: right ventricle weight/ tibial length; C: chronic hypoxia; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; P: pulmonary artery banding

↓ - significant decrease in the parameter

↑ - significant increase in the parameter

↔ - no significant effect in the parameter

* - in the animal model, the parameter did not significantly worsen

Table S2 – Other results

		SV				TIMP-1				Treadmill distance				RVWT				RV FAC			
Study	Drug(s)	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P	C	M	S	P
1	Urocortin-2										↑										
2	Sacubitril/valsartan			↔	↔																
3	GS-444217						↓	↓													
4	Sodium valproate														↓		↓				
5	Ivabradine		↑	↑	↑						↑	↔	↑*						↔	↑	↑
6	Sunitinib														↓		↓				
6	Sorafenib														↓		↓				
7	Juglone																				
8	Celastrol											↑					↓			↑	↑
9	Dapaglifozine																				
10	Macitentan																↔				
10	Tadalafil																↔				
10	Mac. + Tad.																↔				
11	Neuregulin-1																				
12	GapmeR H19		↑		↑														↑		↑
13	MitoQ													↓			↔				
14	Dichloroacetate														↓						
15	Gallein										↔		↑								
16	Sildenafil		↑		↔		↓		↔												
17	Clorgyline			↔	↔																
18	Dantrolene														↓						
19	RVX208				↑																

SV: stroke volume; RVWT: right-ventricular wall thickness; RV FAC: right-ventricular fractional area change; M: monocrotaline (with or without shunt); S: Sugen 5416/hypoxia; P: pulmonary artery banding

↓ - significant decrease in the parameter

↑ - significant increase in the parameter

↔ - no significant effect in the parameter

* - in the animal model, the parameter did not significantly worsen

Scale for the Assessment of Narrative Review Articles – SANRA

Please rate the quality of the narrative review article in question, using categories 0–2 on the following scale. For each aspect of quality, please choose the option which best fits your evaluation, using categories 0 and 2 freely to imply general low and high quality. These are not intended to imply the worst or best imaginable quality.

Nota: Para a UC de Dissertação/Projecto, todos os seis items devem ser cumpridos em nível 2.

1) Justification of the article's importance for the readership

The importance is not justified. — 0
The importance is alluded to, but not explicitly justified. — 1
The importance is explicitly justified. — 2

2

Página 2, parágrafo 4, "No approved therapy targets the RV(9). This difficulty on the translation from animal models has multiple explanations."

2) Statement of concrete aims or formulation of questions

No aims or questions are formulated. — 0
Aims are formulated generally but not concretely or in terms of clear questions. — 1
One or more concrete aims or questions are formulated. — 2

2

Página 3, parágrafo 1, "We aim to provide a picture of the effect of pre-clinical and clinical therapies on multiple animal models, with a speacial focus on PAB"

3) Description of the literature search

The search strategy is not presented. — 0
The literature search is described briefly. — 1
The literature search is described in detail, including search terms and inclusion criteria. — 2

2

Página 3, parágrafos 2 e 3, "The query used was: (pulmonary hypertension OR pulmonary arterial hypertension) (...) pulmonary artery banding or PTB or pulmonary trunk banding)

4) Referencing

Key statements are not supported by references. — 0
The referencing of key statements is inconsistent. — 1
Key statements are supported by references. — 2

2

Página 6, parágrafo 3, "(...) mechanically constrict the pulmonary trunk, developing RV dysfunction, without affecting pulmonary vessels(30). Therefore, if a drug improves RV function of a PAB animal, that drug likely has a direct cardioprotective action(6).

5) Scientific reasoning

(e.g., incorporation of appropriate evidence, such as RCTs in clinical medicine)

The article's point is not based on appropriate arguments. — 0
Appropriate evidence is introduced selectively. — 1
Appropriate evidence is generally present. — 2

2

Página 2, parágrafo 4, "In fact, in a recent meta-analysis, only 41 out of 522 interventions in animal models (8%) were ineffective(8).

6) Appropriate presentation of data

(e.g., absolute vs relative risk; effect sizes without confidence intervals)

Data are presented inadequately. — 0
Data are often not presented in the most appropriate way. — 1
Relevant outcome data are generally presented appropriately. — 2

2

Página 4, parágrafo 6, "Overall, 63,0% of PH models results agree with the PAB results (40,3% both improve and 22,7% both have no significant effect)."

Sumscore 12

Fig. 1 SANRA - Scale

Nota 1: O ponto 5 diz respeito à contextualização da evidência científica em relação ao tipo de estudo(s) que a produziu (e, idealmente, à qualidade do(s) mesmo(s)). O ponto 6 diz respeito a sustentar as afirmações com os dados quantitativos mais apropriado. A título de exemplo, para cumprir o ponto 5 e o ponto 6, ao invés de afirmar "já foi evidenciado que os testes de alergia às penicilinas apresentam alta especificidade e moderada sensibilidade", dever-se-á indicar "uma revisão sistemática com meta-análise de acuidade diagnóstica evidenciou que os testes de alergia às penicilinas apresentam alta especificidade (valor meta-analítico: 97%; IC95%=94-98%) e moderada sensibilidade (valor meta-analítico: 31%; IC95%=19-46%).

SANRA – explanations and instructions

This scale is intended to help editors assess the quality of a narrative review article based on formal criteria accessible to the reader. It cannot cover other elements of editorial decision making such as degree of originality, topicality, conflicts of interest or the plausibility, correctness or completeness of the content itself. SANRA is an instrument for editors, authors, and reviewers evaluating individual manuscripts. It may also help editors to document average manuscript quality within their journal and researchers to document the manuscript quality, for example in peer review research. Using only three scoring options, 0, 1 and 2, SANRA is intended to provide a swift and pragmatic sum score for quality, for everyday use with real manuscripts, in a field where established quality standards have previously been lacking. It is not designed as an exact measurement of the quality of all theoretically possible manuscripts. For this reason, the extreme values (0 and 2) should be used relatively freely and not reserved only for perfect or hopeless articles.

We recommend that users test-rate a few manuscripts to familiarize themselves with the scale, before using it on the intended group of manuscripts. Ratings should assess the totality of a manuscript, including the abstract. The following comments clarify how each question is designed to be used.

Item 1 – Justification of the article's importance for the readership

Justification of importance for the readership must be seen in the context of each journal's readership.
Consider how well the manuscript outlines the clinical problem and highlights unanswered questions or evidence gaps – thoroughly (2), superficially (1), or not at all (0).

Item 2 – Statement of concrete/specific aims or formulation of questions

A good paper will propose one or more specific aims or questions which will be dealt with or topics which will be reviewed.
Please rate whether this has been done thoroughly and clearly (2), vaguely or unclearly (1), or not at all (0).

Item 3 – Description of the literature search

A convincing narrative review will be transparent about the sources of information on which the text is based. Please rate the degree to which you think this has been achieved. To achieve a rating of 2, it is not necessary to describe the literature search in as much detail as for a systematic review (searching multiple databases, including exact descriptions of search history, flowcharts, etc.), but it is necessary to specify search terms, and the types of literature included. A manuscript which only refers briefly to its literature search would score 1, while one not mentioning its methods would score 0.

Item 4 – Referencing

No manuscript references all statements. However, those that are essential for the arguments of the manuscript – "key statements" – should be backed by references in all or almost all cases. Exceptions could reasonably be made for rating purposes where a key statement has uncontroversial face-validity, such as "Diabetes is among the commonest causes of chronic morbidity worldwide."
Please rate the completeness of referencing: for most or all relevant key statements (2), inconsistently (1), sporadically (0).

Item 5 – Scientific reasoning

The item describes the quality of the scientific point made. A convincing narrative review presents evidence for key arguments. It should mention study design (randomized controlled trial, qualitative study, etc), and where available, levels of evidence.
Please rate whether you feel this has been done thoroughly (2), superficially (1), or hardly at all (0). Unlike item 6, which is concerned with the selection and presentation of concrete outcome data, this item relates to the use of evidence and of types of evidence in the manuscript's arguments.

Item 6 – Appropriate presentation of data:

This item describes the correct presentation of data central to the article's argument. Which data are considered relevant varies from field to field. In some areas relevant data would be absolute rather than relative risks or clinical versus surrogate or intermediate endpoints. These outcomes must be presented correctly. For example, it is appropriate that effect sizes are accompanied by confidence intervals. Please rate how far the paper achieves this – thoroughgoingly (2), partially (1), or hardly at all (0). Unlike item 5, which relates to the use of evidence and of types of evidence in the manuscript's arguments, this item is concerned with the selection and presentation of concrete outcome data.

Fig. 2 SANRA—explanations and instructions document

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Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we

propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

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Tables should be inserted at the end of the manuscript in an editable format. If you use a word processor, build your table in Word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Table captions must be placed immediately before the table. Captions should be preceded by the appropriate label, for example 'Table 1.' Please use only a single paragraph for the caption.

Kindly ensure that each table is mentioned in the text and in numerical order.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material.

Tables which are not according to the above guidelines will cause substantial delay during the production process.

Accessibility

Frontiers encourages authors to make the figures and visual elements of their articles accessible for the visually impaired. An effective use of color can help people with low visual acuity, or color blindness, understand all the content of an article.

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Ensure sufficient contrast between text and its background People who have low visual acuity or color blindness could find it difficult to read text with low contrast background color. Try using colors that provide maximum contrast.

WC3 recommends the following contrast ratio levels:

- Level AA, contrast ratio of at least 4.5:1
- Level AAA, contrast ratio of at least 7:1

You can verify the contrast ratio of your palette with these online ratio checkers:

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Avoid using red or green indicators More than 99% of color-blind people have a red-green color vision deficiency.

Avoid using only color to communicate information Elements with complex information like charts and graphs can be hard to read when only color is used to distinguish the data. Try to use other visual aspects to communicate information, such as shape, labels, and size. Incorporating patterns into the shape fills also make differences clearer; for an example please see below:

Supplementary material

Data that are not of primary importance to the text, or which cannot be included in the article because they are too large or the current format does not permit it (such as videos, raw data traces, PowerPoint presentations, etc.), can be uploaded as supplementary material during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to Figshare for permanent storage and receive a DOI.

Supplementary material is not typeset, so please ensure that all information is clearly presented without tracked changes/highlighted text/line numbers, and the appropriate caption is included in the file. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files.

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- data sheet (Word, Excel, CSV, CDX, FASTA, PDF or Zip files)
- presentation (PowerPoint, PDF or Zip files)
- image (CDX, EPS, JPEG, PDF, PNG or TIF/TIFF),
- table (Word, Excel, CSV or PDF)
- audio (MP3, WAV or WMA)
- video (AVI, DIVX, FLV, MOV, MP4, MPEG, MPG or WMV).

Technical requirements for supplementary images:

- 300 DPIs
- RGB color mode.

For supplementary material templates (LaTeX and Word), see our supplementary material templates.

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- All citations in the text, figures or tables must be in the reference list and vice-versa
- The names of the first six authors followed by et al. and the DOI (when available) should be provided
- Given names of authors should be abbreviated to initials (e.g., Smith, J., Lewis, C.S., etc.)
- The reference list should only include articles that are published or accepted
- Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for article types that allow such inclusions

- For accepted but unpublished works use 'in press' instead of page numbers
- Data sets that have been deposited to an online repository should be included in the reference list. Include the version and unique identifier when available
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Harvard reference style (author-date)

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In-text citations

- For works by a single author, include the surname, followed by the year
- For works by two authors, include both surnames, followed by the year
- For works by more than two authors, include only the surname of the first author followed by et al., followed by the year
- For humanities and social sciences articles, include the page numbers.

Reference examples

Article in a print journal Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Website World Health Organization. (2018). E. coli. https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and dissertations Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

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Reference examples

Article in a print journal Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book Sorenson PW, Caprio JC. "Chemoreception". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Website World Health Organization. E. coli (2018). https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

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Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master’s thesis]. [Chicago (IL)]: University of Chicago

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