PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIAS DA SAÚDE

FERNANDA MARIA BOTTINO VIZZOTTO TORETO

POTENCIAL ATEROGÊNICO DA EXPOSIÇÃO CRÔNICA AOS HERBICIDAS GLIFOSATO E ÁCIDO DICLOROFENOXIACÉTICO (2,4-D) EM RATOS

Presidente Prudente - SP
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Dissertação apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título Mestre em Ciências da Saúde – área de concentração: Ciências da Saúde.

Orientadora:
Profª. Drª. Gisele Alborghetti Nai

Presidente Prudente - SP
2023

Catalogação Internacional na Publicação (CIP)

616.1
T678p

Toreto, Fernanda Maria Bottino Vizzotto.
Potencial aterogênico da exposição crônica aos herbicidas glifosato e ácido diclorofenoxiacético (2,4-D) em ratos / Fernanda Maria Bottino Vizzotto Toreto; orientadora Gisele Alborghetti Nai. – Presidente Prudente, 2023.
60f.: il.

Dissertação (Mestrado em Ciências da Saúde) - Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2023.
Bibliografia.

1. Doenças cardiovasculares. 2. Estresse oxidativo. 3. Exposição a praguicidas. 4. Herbicidas. I. Nai, Gisele Alborghetti, orient. II. Título.

Bibliotecária: Jakeline Margaret de Queiroz Ortega – CRB 8/6246

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Presidente Prudente, 26 de janeiro de 2023.

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DEDICATÓRIA

Dedico este trabalho a minha família, que por muitas vezes deixei de lado, não compartilhei festividades, momentos pessoais felizes ou tristes, de dificuldade, de lazer ou de descanso, para poder fazer as pesquisas, estudar, escrever, enfim, para cumprir todas as etapas do estudo. Sei que acreditaram e acreditam em mim e por isso cederam seus tempos a meu favor.

Ao meu esposo, Fábio, que me deu um incentivo enorme, por suas palavras de força e coragem, por me acolher quando chorava e pensava em desistir, por sua ajuda com a informática, por me trazer um café e um lanchinho. Seu coração é especial, sua luz é única e suas palavras orientam e acolhem. Seu amor e sua compreensão foram essenciais para que eu chegasse até aqui. Você é uma daquelas pessoas especiais que poucos têm o privilégio de conhecer, e eu sou uma delas, abençoada por ter você. Amor eterno.

Aos meus filhos, Maria Eduarda e André, que também me ajudaram e me acolheram, gostaria que sempre se lembrem que bens materiais podem ser levados ou perdidos, mas que o conhecimento ninguém tira da gente. Ao meu genro Guilherme, integrante recente na família.
Quero dizer o quanto vocês são importantes para mim. São sensacionais. Sou grata pelo amor, força, apoio incondicional, confiança, entendimento, carinho e compreensão de vocês. Amo cada um do seu jeitinho.

Dedico aos meus pais, Antonio e Maria Alcina, "*In memorian*", por proporcionarem condições para meu estudo e realização do meu sonho, tornar-me médica. Sei o quanto foi sacrificante e tenho certeza que de lá de cima estão muito felizes por mais essa minha conquista. Só tenho a agradecer

AGRADECIMENTOS

Momento difícil na vida acadêmica, pois são tantas pessoas envolvidas que será um pecado esquecer alguém.

Vou começar agradecendo à Deus primeiramente, pela vida. Sei que me capacitou e me deu força para concluir cada etapa na minha existência.

A minha orientadora Profª. Drª. Gisele Alborghetti Nai, que lembrou, acreditou e confiou em mim, e muitas vezes deixou de lado seus momentos para me ajudar e orientar neste trabalho. Pela sua paciência e dedicação durante a realização deste projeto, vários e-mails e mensagens de Whatsapp e muitas vezes sem resposta pela aluna.
Seus conhecimentos foram de suma importância e fizeram relevante diferença no resultado deste trabalho. Foi uma honra para mim. Gratidão!

Às acadêmicas Gabriela Hernandes Ribeiro e Maria Eduarda Silva Souza que auxiliaram na investigação, dedicando seu tempo para que o projeto transcorresse bem.

À Profª. Drª. Renata Calciolari Rossi que muito contribuiu supervisionando os nossos feitos.

À Profª. Drª Francis Lopes Pacagnelli e ao Prof. Dr. Leonardo de Oliveira Mendes pelas considerações e orientações durante a minha qualificação que muito contribuíram para o meu crescimento.

À Unoeste que proporcionou ambiente favorável e a todos os funcionários, que de uma maneira ou de outra, nos forneceram condições para desenvolver nosso projeto.

Aos meus amigos que mandavam mensagens de força e coragem e entenderam a minha ausência nas nossas reuniões festivas.

Em especial a minha família, sem ela não seria possível estar aqui, neste momento, escrevendo estas palavras.

“A parte que ignoramos é muito maior que tudo quanto sabemos”
Platão

“Um sonho não vira realidade a partir de mágica. Você precisa de suor, determinação e trabalho duro.”
Collin Powell

RESUMO

Potencial aterogênico da exposição crônica aos herbicidas glifosato e ácido diclorofenoxiacético (2,4-D) em ratos

Introdução: Herbicidas a base de glifosato (GBH) e ácido diclorofenoxiacético (2,4-D) são os herbicidas mais utilizados no Brasil e no mundo. Ambos têm a capacidade de formação de radicais livres, o que pode acarretar em aterogênese. A aterosclerose pode levar a complicações fatais, como infarto do miocárdio ou doença arterial coronariana, infarto cerebral e gangrena de membros inferiores. A aterosclerose é uma inflamação progressiva das artérias, e a exposição a agrotóxicos vem surgindo como seu fator de risco. **Objetivo:** O objetivo deste estudo foi avaliar histologicamente e comparar o potencial aterogênico da exposição crônica por via oral e inalatória ao GBH e ao 2,4-D em ratos, com concentrações semelhantes às utilizadas na lavoura. **Material e métodos:** Foram utilizados 140 ratos Wistar albinos, machos, adultos distribuídos em 14 grupos, 2 grupos controle, 6 grupos expostos ao glifosato e 6 expostos ao 2,4-D (n=10/grupo): CI - Grupo controle inalatório; CO - grupo controle oral; GLI - Grupo exposto à baixa concentração de GBH por inalação; GLO - Grupo exposto à baixa concentração de GBH por via oral; GMI - Grupo exposto à concentração moderada de GBH por inalação; GMO - Grupo exposto à concentração moderada de GBH por via oral; GHI - Grupo exposto à alta concentração de GBH por inalação; GHO - Grupo exposto à alta concentração de GBH por via oral. DLI - Grupo exposto à baixa concentração de 2,4-D por inalação; DLO - Grupo exposto à baixa concentração de 2,4-D por via oral; DMI - Grupo exposto à concentração moderada de 2,4-D por inalação; DMO - Grupo exposto à concentração moderada de 2,4-D por via oral; DHI - Grupo exposto à alta concentração de 2,4-D por inalação; DHO - Grupo exposto à alta concentração de 2,4-D por via oral. Os animais foram expostos a três concentrações de cada herbicida, por via oral e inalatória: $3,71 \times 10^{-3}$ gramas de ingrediente ativo por hectare (g.i.a/ha), $6,19 \times 10^{-3}$ g.i.a/ha e $9,28 \times 10^{-3}$ g.i.a/ha. Os animais foram eutanasiados após 6 meses de exposição. A aorta foi coletada, corada em HEpara análise histopatológica. **Resultados:** Estrias de colesterol foram observadas somente nos animais expostos aos herbicidas ($p< 0,0001$), sem diferença quanto à via de exposição (oral ou inalatória) ($p> 0,05$). Os animais expostos ao GBH apresentaram o dobro de casos com estrias de colesterol do que os expostos ao 2,4-D ($p< 0,05$). Não houve diferença significante em relação à espessura da aorta entre expostos e não expostos aos herbicidas ($p> 0,05$). Os animais expostos ao 2,4-D mostram maior dimensão fractal dos núcleos quando comparados os animais expostos ao GBH e aos do grupo controle ($p< 0,05$). Não houve correlação entre a medida de espessura da parede da aorta e a dimensão fractal dos núcleos ($r= -0,007$, $p= 0,933$). **Conclusão:** Ambos herbicidas apresentam potencial aterogênico e de dano a parede arterial, porém este é maior na exposição ao GBH.

Palavras-chave: doenças cardiovasculares, exposição a praguicidas, exposição ocupacional, toxicidade crônica.

ABSTRACT

Atherogenic potential of chronic exposure to glyphosate and diclophenoxyacetic acid (2,4-D) herbicides in rats

Introduction: Glyphosate-based herbicides (GBH) and dichlorophenoxyacetic acid (2,4-D) are the most used herbicides in Brazil and worldwide. Both have the ability to form free radicals, which can lead to atherogenesis. Atherosclerosis can lead to life-threatening complications such as myocardial infarction or coronary artery disease, cerebral infarction, and gangrene of the lower limbs. Atherosclerosis is a progressive inflammation of the arteries, and exposure to pesticides has emerged as a risk factor. **Objective:** The aim of this study was to histologically evaluate and compare the atherogenic potential of chronic oral and inhalation exposure to GBH and 2,4-D in rats, at concentrations similar to those used in crops. **Material and methods:** 140 adult male albino Wistar rats were used, distributed into 14 groups, 2 control groups, 6 groups exposed to glyphosate and 6 exposed to 2,4-D (n=10/group): CI - Control group inhalation; CO - oral control group; GLI - Group exposed to low concentration of GBH by inhalation; GLO - Group exposed to low concentration of GBH orally; GMI - Group exposed to moderate concentration of GBH by inhalation; GMO - Group exposed to moderate concentration of GBH orally; GHI - Group exposed to high concentration of GBH by inhalation; GHO - Group exposed to high concentration of GBH orally. DLI - Group exposed to low concentration of 2,4-D by inhalation; DLO - Group exposed to low concentration of 2,4-D orally; AMD - Group exposed to moderate concentration of 2,4-D by inhalation; BMD - Group exposed to moderate concentration of 2,4-D orally; DHI - Group exposed to high concentration of 2,4-D by inhalation; DHO - Group exposed to high concentration of 2,4-D orally. The animals were exposed to three concentrations of each herbicide, orally and by inhalation: 3.71×10^{-3} grams of active ingredient per hectare (g.i.a/ha), 6.19×10^{-3} g.i.a/ha and 9.28×10^{-3} g.i.a/ha. Animals were euthanized after 6 months of exposure. The aorta was collected, HE stained for histopathological analysis. **Results:** Fatty streaks were observed only in animals exposed to herbicides ($p< 0.0001$), with no difference regarding the route of exposure (oral or inhalation) ($p> 0.05$). Animals exposed to GBH had twice as many cases with cholesterol streaks as those exposed to 2,4-D ($p<0.05$). There was no significant difference regarding the thickness of the aorta between exposed and non-exposed to herbicides ($p> 0.05$). Animals exposed to 2,4-D show greater fractal dimension of nuclei when compared to animals exposed to GBH and those in the control group ($p< 0.05$). There was no correlation between the measurement of aortic wall thickness and the fractal dimension of the nuclei ($r= -0.007$, $p= 0.933$). **Conclusion:** Both herbicides have atherogenic potential and damage to the arterial wall, but this is greater when exposed to GBH.

Keywords: cardiovascular diseases, exposure to pesticides, occupational exposure, chronic toxicity.

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ATEROGÊNESE ASSOCIADA EXPOSIÇÃO CRÔNICA A HERBICIDAS A BASE DE GLIFOSATO E DE ÁCIDO DICLOROFENOXIACÉTICO (2,4-D) EM RATOS

Título resumido: **ATEROSCLEROSE E HERBICIDAS**

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O trabalho está apresentado sob a forma de artigo, segundo as normas do periódico o qual será submetido: *Atherosclerosis*, Fator de impacto 5.162, Classificação Qualis A1.

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ANEXOS

ANEXO A – APROVAÇÃO DO TRABALHO PELA COMISSÃO DE ÉTICA EM USO ANIMAL (CEUA) DA UNIVERSIDADE DO OESTE PAULISTA - UNOESTE

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PEIC - Programa Especial de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"COMPARAÇÃO ENTRE O POTENCIAL ATEROGÊNICO MEDIANTE A EXPOSIÇÃO CRÔNICA AOS HERBICIDAS GLIFOSATO E ÁCIDO DICLOFENOXIACÉTICO (2,4-D) EM RATOS"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **6724** e tendo como participante(s) **FERNANDA MARIA BOTTINO VIZZOTTO TORETO (discente), GABRIELA HERNANDES RIBEIRO (discente), MARIA EDUARDA SILVA SOUZA (discente), RENATA CALCIOLARI ROSSI (docente), GISELE ALBORGHETTI NAI (orientador responsável)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 10/03/2021.

MATERIAL ARMAZENADO/DOADO

Protocolo(s)	Data Aprovação	Armazenado (local)	É doação	Detalhes armazenamento
3761	17/05/2017	UNOESTE	NÃO	Laboratório de Anatomia Patológica e Citopatologia - Campus I
5684	13/11/2019	UNOESTE	SIM	Laboratório de Anatomia Patológica e Citopatologia - Campus I

Presidente Prudente, 11 de Março de 2021.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CPDI

Prof ª Dra. Adriana Falcó de Brito
Coordenadora da CEUA - UNOESTE

ANEXO B – NORMAS DE PUBLICAÇÃO DA REVISTA CIENTÍFICA: ATHEROSCLEROSIS

ATHEROSCLEROSIS
International Journal for Research and Investigation on Atherosclerosis and Related Diseases

Atherosclerosis is a fully electronic journal, all manuscripts are to be submitted via the internet. To submit your paper online, click on the link https://www.editorialmanager.com/ath/default.aspx.

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- **Translational Research Papers** reporting results of research from both bench-to-bedside and bedside-to-bench.

The following word limits apply: abstract **250** words, main text **4000** words (including legends to figures and tables), **5** figures and/or tables in total (authors are encouraged to include additional figures and tables as Supplementary Material) and a maximum of **50** references. Flexibility on word count may be offered after discussion with the Editor.

Methodology papers. They describe novel methods or innovative modifications and applications of existing methods for epidemiological, clinical or experimental research on atherosclerosis or vascular biology. The following word limits apply: abstract **150** words, main text **3000** words (including legends to figures and tables), **3** figures/tables in total and a maximum of **25** references.

Rapid Communications. Atherosclerosis welcomes submissions of manuscripts previously rejected by high-quality journals because of priority reasons as Rapid Communications. Please submit your manuscript together with a cover letter, the reviewers' comments and your rebuttal indicating any revisions made to the manuscript via the journal submission system (https://www.editorialmanager.com/ath) by choosing **Rapid Communication** as the article type. Your manuscript will be assessed by the Editor in Chief and Co-Editors,

who will decide **within one week** whether the paper is accepted or not, with or without any revision.

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Conference reports. Conference reports are accepted for publication in our Journal and should be structured as follows: **1)** authors and contact details (postal address of all authors and email address of corresponding author); **2)** name of the conference and name of the organizing national atherosclerosis society); **3)** conference dates and venue, and website address if available; **4)** name(s) of conference President(s); **5)** topics covered by the conference as bullet points; **6)** conference highlights (prosa or bullet points); **7)** conflict of interest statement with respect to the congress (e.g. sponsorship). **1** one-column width figure is allowed. **The word count of the entire report (items 1 through 6) shall not exceed 450 words.**

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[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88), Zenodo, March 25, 2020. https://doi.org/10.5281/zenodo.3727209.

[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. http://dx.doi.org/10.17632/xwj98nb39r.1.

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Atherosclerosis policy on the use of proper terminology when referring to intima-media thickness (IMT)

Atherosclerosis has recently embraced a new editorial policy to clarify the use of proper terminology when referring to intima-media thickness (IMT): **IMT should be referred to as "arterial injury" or "arteriopathy", not atherosclerosis.** For more details, please see the following letter to the editor and reply published in Atherosclerosis

"IMT is not atherosclerosis", Spence
2020 (https://doi.org/10.1016/j.atherosclerosis.2020.09.016) .

"Carotid intima-media thickness should not be referred to as subclinical atherosclerosis: A recommended update to the editorial policy at Atherosclerosis", Raggi and Stein 2020 (https://doi.org/10.1016/j.atherosclerosis.2020.09.015) .

Submission Checklist

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SPECIAL GUIDELINES

Clinical Trials

The International Committee of Medical Journal Editors (ICMJE) defines a clinical trial as any research project prospectively assigning human participants to intervention or comparison groups to study the cause-and-effect relationship between an intervention and a health outcome. Interventions include but are not limited to drugs, surgical procedures, devices, behavioral treatments, process-of-care changes, and the like. All manuscripts reporting clinical trials, must include a copy of the trial protocol including the complete statistical analysis plan, a flow diagram (CONSORT flow diagram), and a completed trial checklist (the CONSORT checklist and template flow diagram can be found at http://www.consort-statement.org/. The trial registration number must be included on the title page of the manuscript reporting a registered clinical trial and in the Materials and methods section. Failure to do so will prevent entry to the peer review process.

Registration of clinical trial

Registration in a public trials registry is a condition for publication of clinical trials in Atherosclerosis in accordance with International Committee of Medical Journal Editors (ICMJE) recommendations. Trials must be registered at or before the onset of patient enrolment. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) will not require registration.

Clinical trial results

In line with the position of the ICMJE, Atherosclerosis will not consider results posted in the same clinical trials registry in which primary registration resides to be prior publication if the results posted are presented in the form of a brief structured (less than 500 words) abstract or table. However, divulging results in other circumstances (e.g., investors' meetings) is discouraged and may jeopardise consideration of the manuscript. Authors should fully disclose all posting in registries of results of the same or closely related work.

Randomized control trials

Reports of randomised trials must conform to CONSORT 2010 guidelines. All manuscripts reporting randomized clinical trials, must include a copy of the trial protocol including the complete statistical analysis plan, a flow diagram (CONSORT flow diagram), and a completed trial checklist (the CONSORT checklist and template flow diagram can be found at http://www.consort-statement.org).

Guidelines for genetic association papers

Atherosclerosis is interested in publishing genetic association papers that present data that is novel, statistically robust, clinically relevant and that add significantly to the field. Authors are advised to follow the reporting guidelines outlined in the STREGA Statement (http://www.strega-statement.org) [1], and to achieve this, the following criteria should be met.

1. All the following aspects should be addressed appropriately and Methods used should be reported:

a) Population stratification should be addressed in case of admixed populations;

b) Test on Hardy-Weinberg-Equilibrium must be carried out and the p value reported;

c) LD-structure between SNPs (if multiple SNPs are reported) must be presented;

d) Genotyping errors / call rate must be reported;

e) Appropriate correction for multiple testing (if multiple independent SNPs are reported) must be included;

f) Possible relatedness between studied subjects must be documented and addressed if present.

2. All papers must include a power calculation to estimate the effect the size the study has the power to detect, based on sample size and minor allele frequency of the included SNPs. If power calculations are not included the paper is likely to be rejected without review. It should be stated whether or not power calculations were performed before or after study completion. Comment: The study should have an adequate sample size. Ideally, power calculations should have been performed before conducting the study since post-hoc power calculations are often a self-fulfilling prophecy. It should be stated whether or not power calculations were

performed before or after study completion. Several programs are available to perform power and/or sample size calculations for genetic association studies, e.g. the "Genetic Power Calculator" (http://pngu.mgh.harvard.edu/~purcell/gpc) [2], and see table 1 below. Sample size and /or Power calculations on two-stage designs can be calculated e.g. by using the program CATS (http://www.sph.umich.edu/csg/abecasis/CaTS) [3] for case-control studies and QpowR (https://www.msu.edu/~steibelj/JP_files/QpowR.html) for studies on quantitative traits. Since genetic association studies often involve more complex study designs involving meta-analysis or several replication stages, simple answers on required sample sizes cannot be given. Authors are advised, however, to keep this issue in mind and give a good rationale, if the study is clearly underpowered.

3. For any novel association a replication study must be included in the submitted manuscript. Any novel association not including a replication study may be rejected without review. Comment: The presentation of novel association results requires replication in most cases, if appropriate replication studies exist. However, if the first study has already an appropriate sample size (considering that very large studies with several thousands of individuals are available) and if the results show a strong association, it might not be necessary to provide a replication. Furthermore, giving additional evidence from other sources could replace replication studies, if they are convincing, e.g. results from functional experiments. Meta-analysis on the discovery stage or other outstanding studies do also not require replication in every case, but it should be clear that these are exceptional cases and have to discussed in that way to be acceptable for publication.

4. For any association study replicating a previously published finding, there should be sufficient novelty to add significantly to the literature. This could include confirming the effect size in a different ethnic group, or extending the association observations to additional intermediate traits or disease groups. Any study not having sufficient novelty is likely to be rejected without review.

5. We require all SNPs to have their designated RS number and for the numbering of base pair changes and amino acid changes and gene symbols to be using agreed nomenclature. For example see the following website: http://www.hgvs.org/mutnomen.

6. Generally, authors should present the rationale as to why gene regions and SNPs have been selected. Association studies using SNPs where previous studies have demonstrated that the base change has an effect on protein function or gene expression will be favored over those using SNPs where no functionality has been previously determined. Studies using a tagSNP approach will also be considered, where these add additional data to the already known variations, in order to further explain observed associations.

References

[1] Little J et al: Strengthening the Reporting of Genetic Association Studies (STREGA): an extension of the STROBE statement. PLoS Med. 2009 Feb 3;6(2):e22.

[2] Purcell S, et al. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 2003, 19(1):149-150.

[3] Skol AD et al. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet (2006) 38:209-13.

In the following table, some sample sizes are given, calculated from the "Genetic Power Calculator", assuming an alpha-level of = 0.05, an additive inheritance model, an assumed prevalence of disease of 30% and a power of 80% for a balanced case-control study (1:1 case:control ratio) for varying minor allele frequencies (MAF) and genetic relative risks (GRR). Relative risks of between 1.1 and 1.3 are in the range that can be expected in genetic association studies on complex diseases.

MAF	GRR assumed per Allele	Cases required in a balanced design
0.01	1.1	40000
	1.3	4700
	1.5	1800
0.05	1.1	8400
	1.3	1000
	1.5	380
0.1	1.1	4500
	1.3	500
	1.5	200
0.2	1.1	2500
	1.3	300
	1.5	125
0.3	1.1	2000
	1.3	250
	1.5	100
0.4	1.1	1700
	1.3	230
	1.5	100

Guidelines for meta-analyses

In principle, literature-based meta-analyses should be reported in that way, that any interested researcher is able to reproduce the results. To ensure this, authors are strongly advised to follow the guidelines listed below and are further encouraged to use the PRISMA (http://www.prisma-statement.org/PRISMAStatement/Default.aspx) and the MOOSE statements (http://jama.ama-assn.org/cgi/content/full/283/15/2008) as a guide. Therefore, as much information as needed should be provided. However, for the average reader only the most mandatory information should be reported in the main paper with additional information given in the Supplementary Material.

1. Specification of objective and primary study outcome. If there are previous meta-analyses on the same outcome available, the authors should specify clearly the differences and added value of their meta-analysis in a separate section ("Added value to previous meta-analysis on the same topic").

2. Detailed specification of search strategy, study selection strategy (including approaches to reach unpublished studies) and eligibility criteria for studies. It is highly recommended to use a graphical Flow Chart (templates available at http://www.prisma-statement.org/PRISMAStatement/Default.aspx).

3. Description of possible sources of bias and confounding and strategies to prevent them. This includes:

- Bias in individual studies
- Bias across studies (e.g. publication bias, selective reporting within studies)
- Quality and comparability of studies (study types, study outcomes, sample size)

4. Description of Statistical Methods:

- What is the primary summary measure (Difference in Mean, OR, etc.)? How was it extracted from the individual studies (e.g. calculated from raw numbers or tables or taken as reported)
- Methods to assess heterogeneity and bias
- Methods used for the combined analysis (fixed effects, random effects) including a rationale for using this method.

5. Reporting of results:

- Individual study characteristics (including sample size, study type, population/ethnicity, primary outcome, reference)
- Individual study results (effect estimates including confidence intervals or standard errors). Graphical presentations is preferred (Forest plots).
- Meta-analysis results: Combined effect estimate, confidence intervals, some measure of heterogeneity, results of bias assessment (preferably using graphical presentations, e.g. Funnel plot)

6. Additional for meta-analysis of genetic association studies: meta-analysis on a single SNP with certain selected outcomes suffer from the problem that they completely ignore the other genetic variability within a certain gene region. Many of these meta-analyses also completely ignore already available results from genome-wide association (GWA) studies on the investigated outcomes. These GWA studies might not have studied the very SNP of interest but highly correlated ones in the

same genetic region which can add valuable information to the meta-analysis. The authors must either discuss the findings from these GWAS or - even much better - approach the authors from these GWAS for a lookup of the meta-analyzed SNPs. Meta analyses that do not cover these issues will be rejected without review. Furthermore, these studies have to report the following information:

- Specification of the genes / polymorphisms (rs numbers) and rationale for selection of the specific polymorphisms
- Genotyping methods in each individual study
- Genotype characteristics (genotyping success rate, minor allele frequency, frequencies of genotypes, Hardy-Weinberg-equilibrium).