THIRD EDITION NOVEMBER 2015

RAMS

Radboud Annals of Medical Students

- Complete Human Head Transplantation before the End of this Decade
- Inflammatory Mediators Inhibit Osteophyte Formation
- The Biggest Walking Event in the World: a Source of Great Research
- Overweight Women at Increased Risk for Breast Cancer Recurrence

Radboudumc

COLOPHON

Edition	Third
Date of publication	November 2015
Place of publication	Nijmegen

CONTACT
89 SOOS - Radboud Annals of Medical Students
Geert Grooteplein Noord 21
6525 EZ Nijmegen
www.ramsresearch.nl

GENERAL BOARD
Lars Gallée (Chair)
Bas Vreugdenhil (Vice-chair)
Barov Sanaan (Treasurer)
Audrey Hollanders (Public Relations)
Sophie van Westendorp (Education)

EDITORS IN CHIEF
David Wolthuis
Cas van der Made
Anna van Boekel

EDITORS
Kim Cortenbach
Jeroen Hol
Jasper Maters
Shelley Dalloyaux
Jasper Kooijman
Ludo van de Linde
Marije Kaan
Gerben Lassche
Ellyze van Asbeck
Bas ten Elzen

CHIEF DESIGN
Jules Janssen Daalen

PHOTOGRAPHER
Esther Simons

SUPERVISORY BOARD
dr. Janiëlle van Alfen-van der Velden
Erwin Beelen (Assessor Medical Faculty)
mr. Bob de Jonge
dr. Jur Koksma
dr. Dirk Schubert

ADVISOR
Michiel Schoenaker

All authors granted written permission for publication.

FROM THE EDITORIAL BOARD

Life is the most beautiful but at the same time hardest thing there is. It is a challenge and a continually changing experience. By practicing medicine we aim to save lives, but also improve the quality of life. Scientific research helps us in this quest. Both in science and in medicine we sometimes have to make difficult decisions. It is not always clear what is best to do, and sometimes extreme thoughts and hazardous experiments are needed to progress. Such as the neurologist who claims he is able to perform a head transplantation. Most people will think he his is mad and that what he is suggesting is absolutely impossible. But who knows? Is that not just what they said to the first person who claimed the world was round?

Therefore sometimes we will have to 'think out of the box' and be brave enough to take the risk. As a toddler learns not to touch something hot because it burns and is painful, we will also learn from our faults. Failures and mistakes are not easy to accept but will eventually help us to improve and progress in whatever we do. In writing them down not only you but also everyone who reads it will learn from it, hopefully preventing mistakes being made twice.

We are able to cure and treat many different diseases but there will always be new challenges. The focus is now changing from curing disease to preventing it. The question is how can we live our lives in such a way that we stay healthy for as long as possible? This one vital question leads to numerous sub questions. Why is it that some people become ill and others stay healthy? What can we do to prevent illness and disease? Would it be possible to prevent diseases such as breast cancer, diabetes or cardiovascular diseases just by eating or not eating certain foods or by walking for half an hour a day?

As prevention is becoming a more and more important aspect of health care, research in preventive medicine is growing. One of the articles in this edition discusses the relationship between disease-free survival after breast cancer and BMI. Moreover in an interview with Professor Maria Hopman you will read about how she combines preventive medicine with her research during the famous Nijmegen Four Day Marches. However, not only is lifestyle important in preventive medicine, but also genetic variants and polymorphisms are becoming more and more relevant; as you can read in an article in this issue discussing the different rates of drug excretion in patients. Here the article suggests that the speed medication is excreted, compared with the general population, depends on the genetic make-up of each individual patient. Research into this kind of genetic variation will help to improve our prescription of medication, which in turn will hopefully reduce the use of ineffective medicine and/or its side effects.

Whilst reading this edition of RAMS you will not only find all of the aforementioned articles but even more interesting and extremely inspiring topics. Today you are the reader....but who knows, maybe one day you will be publishing your own great discoveries!

Anna van Boekel
Editor-in-Chief

INDEX

EFFECT OF BODY MASS INDEX ON RECURRENCE IN BREAST CANCER PATIENTS

R.T.J. Billekens[1], H.J. van Elst[1], S.E. Klarenbeek[2], M.M.P. Munsters[1], B.J.W. Stalmeier[1]

Corresponding author: M.M.P. Munsters (mart.munsters@student.ru.nl)

[1] Bachelor Student, [2] Master Student - Biomedical Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands

N.B. All authors contributed equally to this article

ABSTRACT:

BACKGROUND: Breast cancer is the most common cancer among European and North American or "Western" women. More than half of all patients diagnosed with breast cancer are obese. An association between obesity and risk of primary breast cancer has already been established and a Body Mass Index (BMI) higher than 35 is associated with worse disease-free survival. As weight gain is common during treatment, the percentage of obese patients is even larger after becoming disease-free.
OBJECTIVE: To examine if postmenopausal Western breast cancer patients are more susceptible to cancer recurrence when they are overweight or obese.
METHODS: We performed a systematic literature review and meta-analysis. We searched PubMed and Embase for articles related to recurrence, breast cancer and obesity. We excluded articles when no full text was available and articles which did not use relative risk (RR) as outcome measure. Reviews were not immediately excluded; instead we searched their references for primary studies.
RESULTS: The search yielded seventeen articles, after critical appraisal we included four studies in our meta-analysis. All included studies reported a positive association between risk of breast cancer recurrence and BMI. Compared to normal-weight women, overweight and obese women have a higher risk of recurrence. Our results show a recurrence of 14.3% in non-obese patients, 16.5% in overweight patients and 18.5% in obese patients. This means there are an additional 42 recurrences for every 1,000 obese breast cancer patients. This amounts to a relative risk of 1.13 (95%CI: 1.02-1.24) and 1.27 (95%CI: 1.14-1.41), respectively.
CONCLUSION: Our meta analysis showed that both being overweight and obese are risk factors for recurrence in BC patients. We hypothesise that reducing BMI will lead to lower breast cancer recurrence. Therapies directed at losing weight could become a standard after-treatment procedure for obese people, who are at higher risk for breast cancer recurrence.

WHAT'S KNOWN: Obesity is a medical condition and a growing problem in the Western world, associated with type II diabetes and metabolic syndrome, among other pathologies. An association between obesity and risk of primary breast cancer has been established and a BMI higher than 35 is associated with worse disease free survival.

WHAT'S NEW: Overweight women are at increased risk for recurrence of breast cancer. It is advised to strive for a healthy weight in the treatment of breast cancer and reducing the risk of recurrence.

KEYWORDS: Obesity, breast cancer, recurrence, postmenopausal

Introduction

Breast cancer is the most common cancer among women, especially in the Western population. One in eight Dutch females are diagnosed with breast cancer at some point in their life and 3,200 women die of breast cancer each year. 75% of people diagnosed with breast cancer are over 50 years old [1]. Because of the national screening program for breast cancer smaller tumours are detected and, together with improved treatment, mortality has decreased [2]. The 10 year survival rate for women with breast cancer is 70% and increasing [2,3].

Most patients diagnosed with early stage breast cancer will undergo either a lumpectomy or mastectomy followed by local or systemic therapy, sometimes combined with radiation therapy. The choice of treatment depends on the grade and size of the tumour, involvement of the lymph nodes, hormone sensitivity and general health of the patient [4].

After successful treatment, the probability of breast cancer recurrence varies greatly between individuals. Positive lymph nodes, premenopausal tumours and triple negative tumours are all examples of factors that give poorer prognosis [4,5]. However, the patient has no influence over these factors, unlike their control over their body weight.

Body weight is partly determined genetically, but diet and physical activity have a large influence [5]. A person is considered overweight with a BMI >25 kg/m2 and is considered obese with a BMI >30 kg/m2 as defined by the World Health Organization (WHO). We defined non-obese as a BMI <25 kg/m2. Obesity is a medical condition and a growing problem in the Western world, associated with type II diabetes and metabolic syndrome, among other pathologies [5,6]. An association between obesity and risk of primary breast cancer has been established [2] and a BMI higher than 35 is associated with worse disease free survival [3]. A few potential mechanisms have been proposed: hyperinsulinaemia may help proliferation and invasion of potentially cancerous cells [7]. Adiposity causes higher blood concentrations of oestrogen, which may play an important role in hormone sensitive breast cancer [5]. More than half of all patients diagnosed with breast cancer are overweight or obese and weight gain is common during treatment [3,8].

The purpose of this study is to determine if postmenopausal Western breast cancer patients are more susceptible to cancer recurrence when they are overweight or obese compared to non-obese. All included patients were treated for primary breast cancer. We defined recurrence as a new local tumour or a metastasis. We considered someone overweight or obese as defined by the WHO and patients were considered postmenopausal when the menopause was a natural event. We included only postmenopausal women, because this reduces the confounding effect that hormone levels might have on the study.

Table 2 *Critical Appraisal - RCT, randomised controlled trial; N, number of patients; BMI, two or three group separated by BMI; MR, BMI measured by researcher; CG, control group (normal BMI); LF, Length of follow-up (5-10yr =+ ; >10yr =++); LS, Loss to follow-up <20%; OU, recurrence is primary outcome; +, adequate; -, inadequate or unreported.*

			Criteria					
Study	Design	N	BMI	MR	CG	LF	LS	OU
Ewertz et al, 2012 [9]	RCT	4760	+	-	+	+	+	-
Kamineni et al, 2012 [10]	Cohort	288	+	-	+	++	-	+
Robinson et al, 2014 [11]	Cohort	1155	+	-	+	+	-	+
Sestak et al, 2010 [12]	RCT	4933	+	?	+	-	-	+
Loi et al, 2005 [13]	Cohort	485	+	-	+	+	-	+
Majed et al, 2011 [14]	Cohort	15166	+	+	+	++	-	+

Table 1 *Search Strategy.*

Database	Search
Pubmed	("survival"[Mesh] OR survival[tiab] OR "Mortality"[Mesh] OR "neoplasm Recurrence, Local"[Mesh] OR recurrence[tiab]) AND ("Breast Neoplasms"[Majr] OR breast cancer[ti] OR breast neoplasms[ti] OR breast carcinoma[ti]) AND ("Overweight"[Mesh] OR body weight[tiab] OR obesity[tiab])
Embase	(Exp recurrence/ OR recurrence.ti,ab. OR Cancer recurrence.ti,ab. OR Relapse.ti,ab. OR Exp recidivism/ OR recidivism.ti,ab. OR recidive.ti,ab.) AND (Exp breast tumor/ OR breast tumor.ti,ab. OR breast neoplasm*. ti,ab. OR breast cancer.ti,ab.) AND (Exp obesity/ OR obesity.ti,ab. OR obese.ti,ab. OR overweight.ti,ab.)

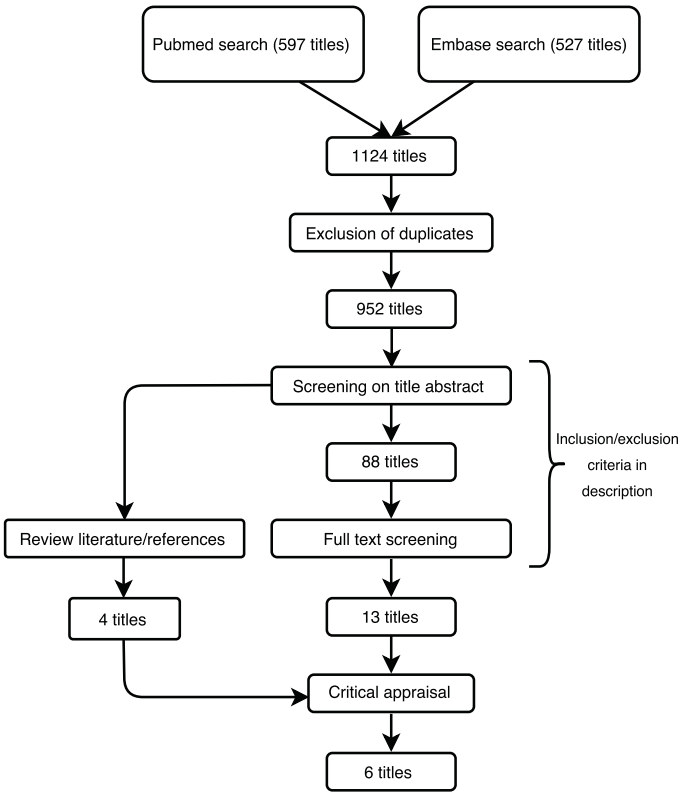


Figure 1 *Flowchart of the search and appraisal process.*
Inclusion criteria: Recurrence as primary outcome; Groups based on BMI.
Exclusion criteria: No full text; studies done before 2005; animal studies; non-western population; no follow-up.

Methods

The terms and synonyms we defined to be used in Embase and PubMed were based on the population: female postmenopausal breast cancer survivors; on the determinants: being overweight or obese; and on the outcome: recurrence. Our search strategy can be found in table 1.

After the search, we first removed duplicates, then we selected articles based on title and abstract and afterwards on full-text screening. We excluded studies if it was an animal study or included a non-western population. Articles were also excluded when we could not access the full text. Reviews were not immediately excluded; we searched their references

Table 3 *Meta-analysis of relative risk of BMI on recurrence of breast cancer - N: number of breast cancer patients; BMI<=25: incidence of recurrence in group with BMI below or equal to 25 during follow-up, same for other groups; RR overweight: relative risk of recurrence of BMI 25-30 group compared to BMI<25 group; RR obesity: relative risk of recurrence of BMI>30 group compared to BMI<25 group; Total: meta-analysis of the studies, weighted by number of patients. Recurrence is higher in patients that have a higher BMI. Both the relative risks for overweight and for obesity are significant.*

Study	N	BMI <=25	BMI 25>=30	BMI >30	RR overweight	RR obesity
Ewertz	4760	16.1%	17.5%	18.3%	1.08[0.94-1.25]	1.14[0.97-1.34]
Kamineni	485	9.8%	10.0%	22.2%	1.03[0.56-1.90]	2.28[1.32-3.92]
Robinson	1155	6.4%	8.6%	12.7%	1.34[0.84-2.14]	1.98[1.25-3.14]
Sestak	4933	15.3%	17.7%	19.4%	1.16[1.00-1.34]	1.27[1.09-1.48]
Total [5]	11333	14.3%	16.5%	18.5%	1.13[1.02-1.24]	1.27[1.14-1.41]

instead and found four additional articles for the critical appraisal. Figure 1 shows a flowchart of how we included articles. All included articles contained either almost only postmenopausal women or stratified data for pre- and postmenopausal women.

Subsequently, we critically appraised the found articles based on the Newcastle-Ottawa scales for cohort studies, with which we compiled our own set of criteria, which was also relevant for randomised controlled trials (RCTs), to appraisethe articles, which can be found in table 2.
Finally, we performed a statistical analysis using the standard functions in the program Cochrane Review Manager, calculating RRs and heterogeneity between the studies. The studies used in this analysis were weighed by the number of patients included in those studies: studies with more patients contributed more to the result of the meta-analysis. By doing this we corrected for the size of the studies.

Results

Our initial search returned 597 articles from PubMed and 527 from Embase. Exclusion, removal of duplicates and inclusion of articles referenced yielded seventeen articles to enter the critical appraisal.This ultimately resulted in six articles to be used in our case report. The following articles were included in the meta-analysis; Ewertz et al. [9], Kamineni et al. [10], Robinson et al. [11] and Sestak et al. [12].
We could not determine the amount of breast cancer recurrences and

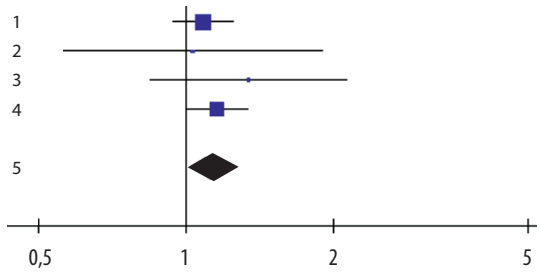


Figure 2 *Forest plot of meta-analysis of relative risks of overweight in breast cancer patients on recurrence. 1- Ewertz et al., 2- Kamineni et al., 3- Robinson et al., 4- Sestak et al., 5- Pooled effect. The overall effect is significant. The studies are homogeneous (P=0.80; I2=0%).*

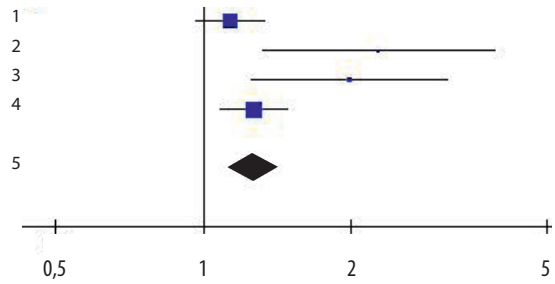


Figure 3 *Forest plot of meta-analysis of relative risks of obesity in breast cancer patients on recurrence. 1- Ewertz et al., 2- Kamineni et al., 3- Robinson et al., 4- Sestak et al., 5- Pooled effect. The overall effect is strongly significant. The smaller studies show a larger effect; the studies are heterogeneous (P=0.02; I2=70%).*

thus relative risk (RR) from the study by Loi et al. [13]; Majed et al. [14] only used contralateral breast cancer as an outcome measure. These studies were excluded from the meta-analysis.

The study of Ewertz et al. is an RCT in which the association between BMI and the risk of recurrence or death in postmenopausal women with breast cancer is examined. The patients received adjuvant tamoxifen or letrozole. Both drugs block oestrogen effects. In this study, 4,760 patients with early stage breast cancer were included. Breast cancer recurrence is a secondary outcome measure, with disease-free survival as primary outcome measure. The study did not find any significant results in outcome measures between treatment with tamoxifen or letrozole.

The study of Kamineni et al. reports that obesity may contribute to poorer breast cancer outcomes. A cohort design was used in which 485 women of over 40 years were included with stage I/II breast cancer. The primary outcome measure is breast cancer recurrence in the ipsilateral breast or distant recurrence after 120 days following the completion of the initial course of therapy.

Robinson et al. associates obesity with poorer invasive breast cancer prognosis. In this cohort study, 1,199 women were recruited (of which 296 were premenopausal) with a mean age of 58 years. The participants completed an enrolment questionnaire (completed between 8 and 58

weeks from diagnosis) and completed an annual follow-up questionnaire every 12 months for 5 years, in which they reported recurrence or new breast cancer. The outcome used is local or distant recurrence, new breast cancer, or death due to breast cancer.

The results of Sestak et al. confirm poorer prognosis of obese women with early stage breast cancer. This study is a double-blind RCT in which postmenopausal women with early-stage breast cancer were randomly assigned to receive anastrozole, which also blocks oestrogen effects, tamoxifen or the combination in a double-blind fashion. The impact of BMI on recurrence and the relative benefit of anastrozole versus tamoxifen according to baseline BMI were investigated. The primary outcome measure is recurrence. Obesity is not the primary determinant, but obesity was taken into account and included in the results.

Table 3 shows the results of the meta-analysis that was carried out. Our results are not weighted for person-years Recurrence was found in 14.3% of patients with a BMI lower than 25 (healthy group), in 16.5% of patients with a BMI between 25 and 30 (overweight group), and in 18.5% of patients with a BMI higher than 30 (obese group). The RR of recurrence of the overweight group compared to the healthy group was significantly higher (RR= 1.13; 95%CI[1.02-1.24]; p=0.02). The RR in the obese group compared to the non-obese group was even higher and strongly significant (RR=1.27; 95%CI[1.14-1.41]; p<0.0001).

Figures 2 and 3 show a visual representation of the meta-analysis. It shows that the studies are homogeneous for overweight (P=0.80; I2=0%) but not for obesity (P=0.02; I2=70%); the smaller studies show a larger effect of obesity on breast cancer recurrence.

Discussion

According to the meta-analysis, the relative risk of recurrence in the overweight group is significantly higher compared to the normal BMI group (RR=1.13; 95%CI=[1.02-1.24]). For the obese patients, we see similar results (RR=1.27; 95%CI=[1.14-1.41]). Being overweight or obese is a potential risk factor for breast cancer recurrence based on these results.

The main strengths of our systematic review lie with the extensiveness of the search strategy and the critical appraisal. We searched both Pubmed and Embase using a multitude of synonyms and in addition to that, we also searched the references of a number of systematic reviews. As a result, the chance of missing relevant studies was minimised. We identified one weakness in our search strategy: studies from before 2005 were not included because of the possibility of dated results. In retrospect, we should not have used this exclusion criterion because we did not have evidence to assume that results from studies before 2005 and after 2005 differ from each other. Besides that, if we had included studies from before 2005 in our search, we probably would have had included more articles in our analysis.

A limitation of our study lies with the published literature. Ideally, we would have preferred all the studies included to have measured the BMI of the patients after treatment, as chemotherapy may induce weight gain [15]. Had we known the weight of the women after treatment, we could potentially have demonstrated a larger effect than we have now. Secondly, three of the four included studies did not report recurrence for premenopausal and postmenopausal patients separated. However, the median age of women included was far beyond menopause in these studies. Ewertz et al. had a median age of 62, Kamineni et al. had a percentage of women below 50 of only 22%, Robinson et al. had a mean age of 58. Only Sestak et al. exclusively included postmenopausal women. Considering that some premenopausal women were included in our analysis, our found effect may be smaller than it is in actuality. This is because obesity has been found to be a risk-lowering factor for premenopausal women [16]. Finally, we would have appreciated conformity in the outcome measures. As Kamineni et al. did not consider contralateral breast cancer as recurrence, our results might have been skewed. We had to exclude Majed et al. from our meta-analysis as they only looked at contralateral breast cancer recurrence.

A part of the heterogeneity between the included studies can be explained due to the difference in outcome measures. Another important factor in the heterogeneity is the difference in follow-up; the longer the follow-up, the larger the amount of recurrences. Ewertz had a median follow-up of 8.7 years, Kamineni used a follow-up of 10 years, Robinson used 5 years and Sestak had a median follow-up of 8.3 years. This implies that in the Kamineni study, the reported number of recurrences should be higher than in the other studies. This is true for a BMI >30.

A factor that could have confounded our results is the medication used in the randomized controlled trials we included. Letrozole was more effective than tamoxifen in all BMI groups in reducing breast cancer recurrences. Anastrozole was more effective as well, but had a greater effect in thin women than in obese women [9,12]. As such, the group receiving anastrozole may have contributed to a larger effect of obesity in our meta-analysis.

As all included studies reported a greater risk of breast cancer recurrence in obese women, the conclusion that obesity is in fact a risk factor for breast cancer recurrence is valid. The actual impact might slightly differ from our meta-analysis, as we identified both factors that might reduce or that might increase the actual effect. Due to confounding and variation in literature, it is hard to estimate the actual size of the effect. However, it is clear that there is an effect, as the meta-analysis shows a very strongly significant result and the relative risk of 1.27 is a good estimation of the effect.

According to several studies [16,17], obesity is a risk-reducing factor in premenopausal women whereas it increases the risk of contracting breast cancer in postmenopausal women. This is probably due to a hormonal mechanism that changes during menopause [18]. Similar to our study, Loi et al. found a positive correlation between obesity and breast cancer recurrence in postmenopausal women with a hazard ratio of 2.03 (95%CI: 0.99 – 4.21) [13]. In contrast to our results, Majed et al. found obesity to be a protective factor for contralateral breast cancer recurrence in postmenopausal women (RR=0.91; 95%CI: 0.75-1.11)14. This can be explained by the difference in outcome measures: contralateral breast cancer recurrence is only a small part of total recurrence.

We hypothesise that reducing BMI will lead to lower breast cancer recurrence and better life expectancy in general. As such, we would recommend a randomized controlled trial that divides postmenopausal obese breast cancer survivors in two groups; one receiving a weight loss intervention whereas the other group does not. Comparing the recurrence of breast cancer between these groups could show the value of such an intervention and could complement the outcome of our research. The cost of such an intervention programme could be weighed against the beneficial effects. As weight loss without an intervention programme is free of cost, a demonstrated effect in the RCT could add to the recommendation of losing weight. In turn, this could lead to changes in guidelines for postmenopausal breast cancer survivors; weight loss could become a standard after-treatment intervention programme for obese patients, who are highly at risk for a renewed episode of breast cancer, should it be cost-effective. In addition, we would advise medical professionals to better monitor the weight of breast cancer patients, both during

treatment and follow-up.

Conclusion

In conclusion, our results show a recurrence of 14.3% in non-obese patients and 18.5% in obese patients. This means there are an additional 42 recurrences in every 1,000 obese breast cancer patients. Something to keep in mind is that obesity is associated with a broad range of diseases, such as diabetes and cardiovascular diseases. Our results strengthen the recommendation to maintain a "healthy" BMI, preferably below 25, in order to decrease the risk of breast cancer.

Acknowledgements

We would like to thank our supervisors F. de Vegt and M. Broeders for their advice and support. They are both employed at the Department of Health Evidence, within the section of cancer epidemiology research.

References

1. RIVM. Bevolkingsonderzoek Borstkanker veel gestelde vragen. Available via: http://www.rivm.nl/Onderwerpen/B/Bevolkingsonderzoek_borstkanker/Veelgestelde_vragen/Borstkanker (9 maart 2015)
2. LETB. Landelijke evaluatie van bevolkingsonderzoek naar borstkanker in Nederland. Optima Grafische Communicatie, Rotterdam. April 2014 p.38 ISBN 978-94-6169-494-2
3. Cantarero-Villanueva I, Galiano-Castillo N, Fernandez-Lao C, Diaz-Rodriguez L, Fernandez-Perez AM, Sanchez MJ, et al. The Influence of Body Mass Index on Survival in Breast Cancer Patients. Clinical breast cancer. 2015;15(2):e117-23. Epub 2014/12/17. 4.
4. Uptodate. Overview of the treatment of newly diagonsed non metastatic breast cancer. Available via: http://www.uptodate.com/contents/overview-of-the-treatment-of-newly-diagnosed-non-metastatic-breast-cancer?source=machineLearning&search=breast+cancer&selectedTitle=1~150&sectionRank=1&anchor=H528513260#H528513260. Consulted on: 21 maart 2015
5. Azrad M, Demark-Wahnefried W. The association between adiposity and breast cancer recurrence and survival: A review of the recent literature. Current nutrition reports. 2014;3(1):9-15. Epub 2014/02/18.
6. James FR, Wootton S, Jackson A, Wiseman M, Copson ER, Cutress RI. Obesity in breast cancer - What is the risk factor? European journal of cancer (Oxford, England : 1990). 2015. Epub 2015/03/10.
7. Stoll BA. Adiposity as a risk determinant for postmenopausal breast cancer. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000;24(5):527-33. Epub 2000/06/13.
8. Vagenas D, DiSipio T, Battistutta D, Demark-Wahnefried W, Rye S, Bashford J, et al. Weight and weight change following breast cancer: evidence from a prospective, population-based, breast cancer cohort study. BMC cancer. 2015;15(1):28. Epub 2015/02/01.
9. Ewertz M, Gray KP, Regan MM, Ejlertsen B, Price KN, Thurlimann B, et al. Obesity and risk of recurrence or death after adjuvant endocrine therapy with letrozole or tamoxifen in the breast international group 1-98 trial. Journal of Clinical Oncology. 2012;30(32):3967-75.
10. Kamineni A, Anderson ML, White E, Taplin SH, Porter P, Ballard-Barbash R, et al. Body mass index, tumor characteristics, and prognosis following diagnosis of early-stage breast cancer in a mammographically screened population. Cancer causes & control : CCC. 2013;24(2):305-12. Epub 2012/12/12.
11. Robinson PJ, Bell RJ, Davis SR. Obesity is associated with a poorer prognosis in women with hormone receptor positive breast cancer. Maturitas. 2014;79(3):279-86. Epub 2014/08/05.
12. Sestak I, Distler W, Forbes JF, Dowsett M, Howell A, Cuzick J. Effect of body mass index on recurrences in tamoxifen and anastrozole treated women: an exploratory analysis from the ATAC trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(21):3411-5. Epub 2010/06/16.
13. Loi S, Milne RL, Friedlander ML, McCredie MR, Giles GG, Hopper JL, et al. Obesity and outcomes in premenopausal and postmenopausal breast cancer. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2005;14(7):1686-91. Epub 2005/07/21.
14. Majed B, Dozol A, Ribassin-Majed L, Senouci K, Asselain B. Increased risk of contralateral breast cancers among overweight and obese women: a time-dependent association. Breast cancer research and treatment. 2011;126(3):729-38. Epub 2010/09/28.
15. Vance V, Mourtzakis M, McCargar L, Hanning R. Weight gain in breast cancer survivors: prevalence, pattern and health consequences. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2011;12(4):282-94. Epub 2010/10/01.
16. Benedetto C, Salvagno F, Canuto EM, Gennarelli G. Obesity and female malignancies. Best practice & research Clinical obstetrics & gynaecology. 2015. Epub 2015/03/18.
17. Munsell MF, Sprague BL, Berry DA, Chisholm G, Trentham-Dietz A. Body mass index and breast cancer risk according to postmenopausal estrogen-progestin use and hormone receptor status. Epidemiologic reviews. 2014;36(1):114-36. Epub 2014/01/01.
18. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nature reviews Cancer. 2004;4(8):579-91. Epub 2004/08/03.

HUMAN HEAD TRANSPLANTATION: ARE YOU READY FOR HEAVEN?

Jeroen C. Hol, Jasper J. Kooijman

Everyone knows the famous story of Frankenstein: a completely deranged professor, Victor Frankenstein, creates a horrifying 'creature' in his laboratory, essentially creating a living person out of different body parts. Science fiction, you may think, but not for long if it is up to Sergio Canavero. Earlier this year, Canavero, an Italian neurosurgeon, shocked the world by publishing an article on the possibilities of performing a human head transplantation. He is planning to perform the first human head transplant, complete with a functional patient. In his article he states that modern medicine has both the scientific and the technological capabilities to make such an operation possible within two years [1]. Canavero also published a book on the ethical considerations of the procedure [2]. Despite the large opposition and resistance Canavero faces to carry out his ideas, he is planning on using the proceedings from the book to actually realise the project. The project is called HEAVEN: HEad Anastomosis VENture.

Canavero has taken his inspiration from American neurosurgeon Robert White, who performed the first head transplant on living rhesus monkeys [3]. Although controversial, White's research showed that it was possible to perform a cephalic transplantation with the monkey living up to eight days after the surgery, with no complications whatsoever. However, because in the early seventies there was no technology for reconnecting spinal cords, the monkey was paralysed from the neck down. After White's very first demonstration of a successful cephalic transplant, further exploration of this specific research area has long been left unpursued.

Surgical procedure

The procedure will start with the cooling of the body-recipient's brain to a temperature between 12 and 15 degrees Celsius (the so-called hypothermia protocol). Mammals are known to be able to sustain up to an hour without blood flow at these temperatures. There are several ways to cool a brain down, but Canavero prefers the use of commercial cooling helmets, because of their wide availability and constant cooling. The donor's spinal cord will be selectively cooled by custom-built units, perfusing the spinal subdural and epidural spaces with a cold solution at 4 to 15 degrees Celsius.

During the GEMINI (spinal cord fusion) procedure, surgeons cut the spinal cord with a very sharp blade, making a very clean cut that enables the severed axons to be fused together by using substances called fusogens. These fusogens, which are essentially forms of the inorganic polymer polyethylene glycol (PEG), are able to fuse together cell membranes that have been damaged by mechanical injury.

Medical practice

There are various conditions in today's medical practice that cannot yet be treated, despite our current modern technologies and broad medical knowledge. A good example of these illnesses is progressive muscular dystrophy. Some of the patients suffering from this disease are very young, and have well-functioning brains, but an unfavourable prospect of viable therapies in the near future. In his article, Canavero points out that a young patient suffering from an illness like progressive muscular dystrophy, or rare neurological genetic disorders, would best qualify as the first patient for a head transplant, because of the huge suffering. Also tetraplegics, who show a tendency for multi-organ failure, or patients with intractable cancer without brain metastasis would qualify perfectly for a procedure like HEAVEN. Any disorder that leaves the brain intact, and has a severe influence on the patient's body could possibly be treated with this transplantation.

Challenges

As for all types of organ transplants, immunosuppressive drugs are needed. In the case of a head transplant it remains unsure how to solve this problem. Let us take a look at face transplants for example. Face transplants are being performed since the past decade. So far, episodes of acute skin rejection were present in all recipients, all cases have been treated with conventional immunosuppressive regimens, and no cases of chronic rejection have been reported [4]. It remains unsure whether you can compare a face transplant to a head transplant, however, it is strongly anticipated that a head transplant can be controlled with conventional immunosuppressive regimens. Another option would be to achieve drug-free tolerance. For example by resetting the immune system using Alemtuzumab, a monoclonal antibody that targets white blood cells, during the procedure [5].

Figure 1 *The Italian neurosurgeon Sergio Canavero is planning to perform the first human head transplant.*

However, it is projected that the biggest challenge relates to self and psychological tolerance. Some scientists, including Canavero, believe in a mechanistic framework of the human person. They think when the head and brain are transplanted, the mind, consciousness and person are transplanted as well. On the other hand, modern cognitive science shows that our cognition is an embodied cognition, in which the body is a real part in the formation of human self. In case of a head transplant this would lead to problems in incorporating the new body into the existing body scheme and body image. This could lead to severe psychological problems. Similar problems were also seen in cases of face and hand transplants.

Ethical considerations

In his article Canavero hardly addressed the ethical aspects.1 Although technical feasibility of the procedure is not clear, let us assume the procedure is feasible to perform. What are the ethical considerations of such a procedure?

First, the procedure needs approval by a medical ethical committee. Given the nature of the experiment this could be a problem. However, the procedure is not a therapeutic one, like a face transplant for example, but one intended to prolong life, like a heart or lung transplant. Canavero even suggested it could lead to immortality. This difference might play an essential role in accepting the procedure. A procedure intended to prolong life is more likely to be accepted.
There are ethical problems regarding organ donations in general. The most important one is the uncertainty of the procedure: the donor organs could have been useful to someone else that needs an organ to save his or her life. If the procedure fails, the organs are lost and the heart, lungs, liver, or kidneys cannot be given to other patients who are waiting for donated organs. Moreover, the patient is likely to die as well.

Finally, there is one interesting ethical issue left. Instead of a head transplant, the procedure is actually a full body transplant, using the recepient's head and the donor's entire body. Therefore, the donor's gonads will be transplanted as well. There is a problem with the transplant of the donor's gonads and the transmission of genetic inheritance to offspring. This might be a legal problem, since genetic inheritance is protected by law in many countries. It is not certain whether this will be a problem, because the county in which HEAVEN will take place is not known yet.

Conclusion

This paper describes the procedure of the first human head transplant according to the Italian neurosurgeon Canavero. We have looked into the technical and ethical problems which such a procedure would evoke, including: the use of immunosuppressive drugs, psychological tolerance, the uncertainty of the procedure and transplanting gonads. Only one question remains: would you get your head transplanted in the quest for immortality?

References

1. Canavero S. HEAVEN: The head anastomosis venture Project outline for the first human head transplantation with spinal linkage (GEMINI). Surg Neurol Int. 2013. 4: S335-42
2. Canavero S. Head Transplantation and the Quest for Immortality. Amazon; 2014.
3 White RJ, Wolin LR, Massopust LC Jr, Taslitz N, Verdura J. Primate cephalic transplantation: neurogenic separation, vascular association. Transplant Proc. 1971 Mar; 3(1):602-4.
4. Khalifian S, Brazio PS, Mohan R, Shaffer C, Brandacher G, Barth RN, Rodriguez ED. A Facial transplantation: the first 9 years Lancet 2014; 384: 2153–63
5. Friend PJ. Alemtuzumab induction therapy in solid organ transplantation. Transplant Res. 2013 Nov 20;2(Suppl 1):S5

As RAMS aims to enlighten both students and professionals, we are proud to present a new element in our journal: the exam questions. The questions have been taken from exams from different bachelor modules. Find out if you can remember what you learned during the bachelor! The right answers and the number of students who answered correctly can be found further on in this journal.

We challenge you!

EXAM QUESTIONS

Question 1: Which cells produce a lot of interferon gamma?
Module Immunology

a) Almost every cell in the human body
b) Natural killers cells
c) Macrophages
d) Virus-infected cells

Question 2: How much on average is spent on healthcare in the Netherlands per day?
Module Patient-centered care and health care organisation

a) € 20 million
b) € 50 million
c) € 200 million
d) € 500 million

The answers to these questions can be found on page 17 in this journal

OLIVER SACKS, THE MAN WHO TRAVELLED THE MIND, DIES AGED 82

Ludo S. van de Linde

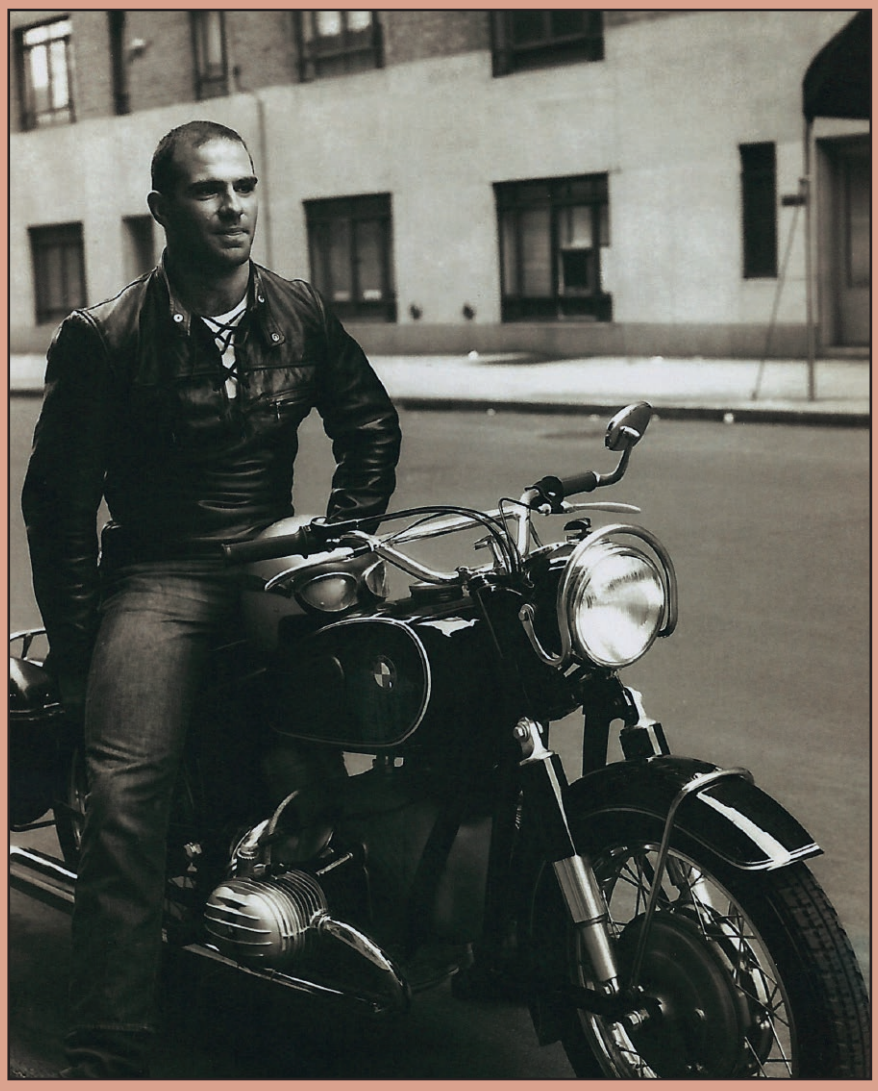
Oliver Sacks, the renowned English-born neurologist and psychiatrist, died at his home in New York on August 30th 2015. He was 82.

Earlier this year he announced that the ocular melanoma, which had been discovered in 2006, had spread to his liver and that he was in the terminal stage of his disease. Upon learning of his impending death, he said that he felt intensively alive and still had things left to do. Among those things was finishing the several books he was writing.

Dr. Sacks was already a prolific writer. He was one of the first to introduce neurology to a greater, non-scientific audience. His books were unpretentious and humorously written accounts of patients he encountered in his practice.

Awakenings (1973) and The Man Who Mistook His Wife for a Hat (1985) are probably among his best known works. In Awakenings he recounts the events that took place after he 'awakened' a group of patients who had been in a catatonic, 'locked-in' state since the encephalitis lethargica epidemic in the 1920s. The book was adapted for film in 1990, starring Robin Williams and Robert De Niro. The Man Who Mistook His Wife for a Hat encompasses a number of compelling patient histories, including the case of Dr. P. who tried to put on his wife's head, assuming it was his hat. The book proved to be so popular, it was adapted into an opera in 1986.

Oliver Sacks was a most remarkable man and doctor, well known for his broad range of interests. A bodybuilding, motorbike riding academic. A neurologist suffering from prosopagnosia. A colourful character indeed.



THE INHIBITORY EFFECT OF IL-1β AND TNF-α ON OSTEOPHYTE FORMATION IN VITRO

Thomas van der Velden[1,2], Esmeralda N. Blaney Davidson[1], Peter M. van der Kraan[1]

Corresponding author: Thomas van der Velden, BSc (thomasvdv@live.nl)

[1] Experimental Rheumatology, Radboud University Medical Centre, Nijmegen, The Netherlands - [2] Bachelor Student Biomedical Sciences

ABSTRACT:

OBJECTIVE: Osteophytes are present in osteoarthritis, but not in rheumatoid arthritis (RA). It has been suggested that osteophytes are absent in RA due to the presence of the inflammatory cytokine tumor necrosis factor-α (TNF-α). We want to investigate whether TNF-α blocks osteophyte formation via the potent osteophyte inhibitor Dickkopf-1 (DKK-1) or via a different signaling pathway. We aimed to determine if interleukin-1β (IL-1β) and TNF-α were able to inhibit formation of growth factor induced osteophytes in vitro.
METHODS: We cultured bovine periosteum as explants. We stimulated them with transforming growth factor-β (TGF-β) and/or bone morphogenetic protein 2 (BMP2) to induce the formation of osteophytes. Then we combined growth factor stimulation with IL-1β or TNF-α to investigate whether these cytokines were capable of blocking osteophyte formation. We performed quantitative polymerase chain reaction for chondrogenesis- and osteogenesis-related genes and DKK-1. Furthermore, we performed microscopic analysis on the histology of the explants.
RESULTS: We observed chondrogenesis in the explants stimulated with TGF-β alone or combined with BMP2. No chondrogenesis was observed histologically in BMP2 stimulated explants. In the explants, exposure to TGF-β alone or combined with BMP2 up-regulated gene expression of aggrecan, but down-regulated osteogenesis related genes and DKK-1. IL-1β down-regulated collagen 2 (Col2) and aggrecan expression in all conditions, while TNF-α was only able to inhibit Col2 and aggrecan in the TGF-β stimulated explants.
CONCLUSION: There was no obvious effect of TNF-α on DKK-1 expression. IL-1β blocked osteophyte formation in growth factor stimulated explants. TNF-α inhibited osteophyte formation in any TGF-β stimulated explant completely, but partly inhibited TGF-β plus BMP2 induced osteophyte formation. With these results, absence of osteophytes in RA cannot be explained only by inhibitory effects of TNF-α through DKK-1.

WHAT'S KNOWN: Osteophytes are present in osteoarthritis, but not in rheumatoid arthritis (RA). It has been suggested that osteophytes are absent in RA due to the presence of the inflammatory cytokine tumor necrosis factor-α (TNF-α) via the potent osteophyte inhibitor Dickkopf-1 (DKK-1).

WHAT'S NEW: Although TNF- α was able to inhibit osteophyte formation in TGF- β stimulated chondrocytes, no effect on DKK-1 expression was found. The absence of osteophytes in RA can therefore not be explained only by inhibitory effect of TNF-α through DKK-1.

KEYWORDS: Osteoarthritis, periosteum, bovine, cytokine, growth factors

Introduction

Osteoarthritis (OA) is a degenerative joint disease involving cartilage, synovium and bone. It is characterized by cartilage breakdown and eventually osteophyte formation [1]. Rheumatoid arthritis (RA) is characterized by inflammation of the synovium and erosion of cartilage and bone [2]. OA and RA are both inflammatory diseases in which the inflammatory cytokines necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) have an important role [3, 4]. However, osteophytes are found in OA, but they are not found in human rheumatoid arthritis (RA). Primarily, we want to determine whether the absence of osteophytes in RA is due to the presence of the inflammatory cytokine TNF-α via the potent osteophyte inhibitor Dickkopf-1 (DKK-1) [5] or via a different signalling pathway. Secondly, we aimed to determine whether IL-1β and TNF-α were able to inhibit formation of growth factor induced osteophytes in vitro, which would explain the absence of osteophytes in RA.

Osteophytes

Osteophytes are newly formed bone spurs at the edge of the joint. They can cause serious medical problems, including restriction of joint movement and pain [6]. However, there is no clear correlation between presence and severity of osteophytes and pain in patients with OA [7]. Osteophytes might also have a positive effect by increasing the joint surface and thereby decreasing the pressure per surface unit, but this is still under debate [6]. Osteophyte formation originates from the periosteum, which is a tissue layer that covers the bone [6]. The process starts with chondrogenesis, followed by formation of cartilage-like tissue called a chondrophyte. Consecutively, the cells undergo hypertrophy and are replaced by bone (osteophyte) [8].

The formation of bone is called osteogenesis and takes place through two different mechanisms: endochondral bone formation (EBF) and intramembranous bone formation (IBF). In OA, osteophytes are mainly formed by EBF, thereby using cartilage as transition phase. In RA models, osteophyte formation through IBS is observed, in which mesenchymal stem cells directly differentiate into osteoblasts [2].

Transforming growth factor β superfamily signaling

In this study we stimulated periosteal cells derived from bovine periost with transforming growth factor β (TGF-β) and/or bone morphogenetic protein 2 (BMP2), both ligands of the transforming growth factor β superfamily, as an in vitro model for osteophyte formation. TGF-β superfamily signalling is involved in many cellular processes, including cell growth, migration, differentiation and apoptosis. TGF-β superfamily signalling is activated when a TGF-β superfamily ligand binds cognate transmembrane receptor kinases.

TGF-β and BMP2 are involved in a variety of development processes including chondrogenesis [8, 9]. In patients with osteoarthritis TGF-β is suggested to have an important role in the pathogenesis, since elevated values of active TGF-β are found in the synovial fluid. Moreover, knee joints which are exposed to TGF-β show changes in OA cartilage and after TGF-β overexpression in the knee joint, osteophyte formation occurred[10]. TGF-β is suggested to initiate the process of the formation of osteophytes by enchondral ossification in OA, whereas BMP2 is important in later phases of this process[8].

Interleukin-1β and tumor necrosis factor-α

The cytokines IL-1β and TNF-α are major mediators of local inflammatory processes in the joint which inhibit cartilage matrix synthesis by chondrocytes. It is also known that IL-1β and TNF-α induce the breakdown of extracellular matrix molecules of articular cartilage [11]. IL-1β not only breaks down extracellular matrix molecules, it also inhibits the synthesis of collagen type II and proteoglycans which are essential components of osteophyte formation [12]. IL-1β is therefore suggested to have an inhibitory effect on osteophyte formation [4].

TNF-α is secreted during inflammation and promotes bone destruction. In RA, TNF-α is suggested to inhibit osteophyte formation via DKK-1, a potent Wingless and Int homolog (Wnt) signalling pathway inhibitor. In human RA joint sections increased DKK-1 levels are found compared to OA sections [5].

Wnt signalling is a key trigger for bone formation. By inhibiting Wnt via DKK-1 in TNF-α transgenic mice, osteophyte formation was absent [5]. In earlier mouse models, we did not find inhibition of osteophyte formation with TNF-α via DKK-1. We found no differences in osteophyte formation in TNF-α knockout mice, compared to wild type mice. We also found that TNF-α does not decrease the incidence of osteophyte formation in RA models. This indicates that the inhibition of Wnt signalling by TNF-α suggested by Diarra et al did not occur in our model [13].

In this project, we will determine whether IL-1β and TNF-α are able to inhibit osteophyte formation. Moreover, we question whether TNF-α has a negative effect on osteophyte formation via DKK-1 as suggested by Diarra et al, since we found inconsistent findings in earlier mice experiments. In these previous experiments we found that TNF-α has a possible role in osteophyte inhibition in RA via another pathway: Smad2/3 phosphorylation. We found low expression of Smad2/3 phosphorylation during streptococcal cell wall induced arthritis [13]. Therefore we want to investigate whether we can reproduce the TNF-α induced osteophyte inhibition via DKK-1.

We hypothesize that IL-1β and TNF-α can block growth factor induced osteophyte formation in vitro. We suggest that TNF-α has no negative effect on osteophyte formation via DKK-1. To investigate our hypothesis, the effect of these cytokines on growth factor induced osteophytes in vitro was determined.

Materials and Methods

Osteophyte formation by culturing periosteal explants

The materials and methods used to obtain periosteal explants, to culture them and to induce osteophyte formation are shown in appendix I. In our first experiment, we determined whether growth factors were able to induce osteophyte formation by histology and by gene expression after 4 and 6 weeks. We stimulated the explants with TGF-β, BMP2 or both. An endpoint of the experiment of 6 weeks was chosen, the same endpoint of culturing performed by O'Driscoll et al [14]. The explant sections used for histology were stained with Safranin O and Fast Green (appendix II). Safranin O is specifically stains products of cartilage including proteoglycans red and Fast Green stains subchondral bone and fibrous tissue blue [15, 16]. We measured the mRNA expression of Glyceraldehyde-3-phospate dehydrogenase (GAPDH), DKK-1, aggrecan, type I collagen (Col1), Col2 and Col10, runt-related transcription factor-2 (Runx2) and osteocalcin to determine the effect of growth factors on the explants. The housekeeping gene GAPDH was used as internal control, since it is expressed continuously and is involved in cell catabolic processes [17]. Cartilage contains the extracellular matrix components Col2 and the proteoglycan aggrecan, whereas bone contains Col1 [18]. Col2 and aggrecan are increased in the early process of chondrogenesis during EBF. Col10 is a marker for late-stage chondrocyte hypertrophy associated with EBF [19, 20]. Runx2 is a master regulator of osteoblast and terminal chondrocyte differentiation and is an accepted early marker for osteogenesis [20, 21]. Osteocalcin is a bone specific protein and therefore a measure for calcification [22]. The relative changes in mRNA expression of these genes in response to growth factors was measured by quantitative polymerase chain reaction (Q-PCR) analysis.

Table 1 *Q-PCR primer list.*

Gene	Forward primer[1]	Reverse primer[1]
Aggrecan	TGAAACCACCTCCACCTTCCATGA	TCAAAGGCAGTGGTTGACTCTCCA
Col1	AGTCAAGAACTGGTACAGAAATTCCAA	CTGGGTACCACCGTTGATAGTTT
Col2	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG
Col10	CCATCCAACACCAAGACACAGT	TGCTCTCCTCTCAGTGATACACCTT
DKK-1	GCAGCAAGTACCAGACCATTGAC	CGACAGGCGAGGCAGATT
GAPDH	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT
OC	CCCAAGAGGGAGGTGTGTGA	GCCGATAGGCTTCCTGGAA
Runx2	TGCACCACCACCTCGAATG	CTTCCGTCGGCGTCAAC

[1] Forward and reverse primers are shown from 3' to 5', OC, osteocalcin

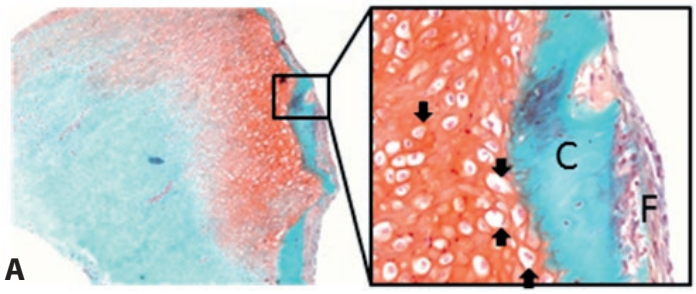
The effect of TNF-α and IL-1β on osteophyte formation

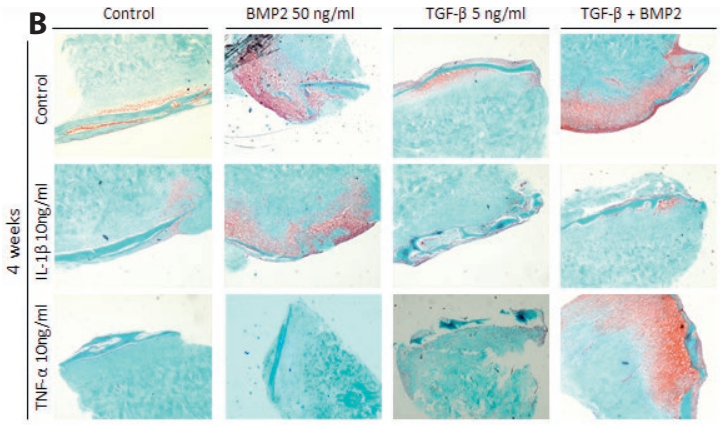
To identify the role of TNF-α and IL-1β on osteophyte formation we stimulated bovine periosteal explants with TGF-β, BMP2 or both, combined with TNF-α and IL-1β exposure. The effects of TNF-α and IL-1β on growth factor induced osteophyte formation were determined by histology and gene expression.

mRNA isolation and Q-PCR

mRNA was extracted from the periosteal explants through different steps (appendix III). Eventually, gene expression of the genes of interest (Aggrecan, Col1, Col2, Col10, DKK-1, GAPDH, osteocalcin and Runx2) (table 1) were determined by Q-PCR. The primer mix consisted of a forward and a reversed primer of each gene.

All primers were obtained from Biolegio BV (Nijmegen, the Netherlands). Q-PCR quantified the results in CT (threshold cycle). The threshold was put at 0.2, which is at the linear part of the curve in the amplification plot. The CT values of the interested genes of a sample were corrected for GAPDH, which leads to the ΔCT value. Then the ΔCT values of the interested genes in stimulated explants were corrected for the unstimulated control explants which eventually leads to a ΔΔCT value.





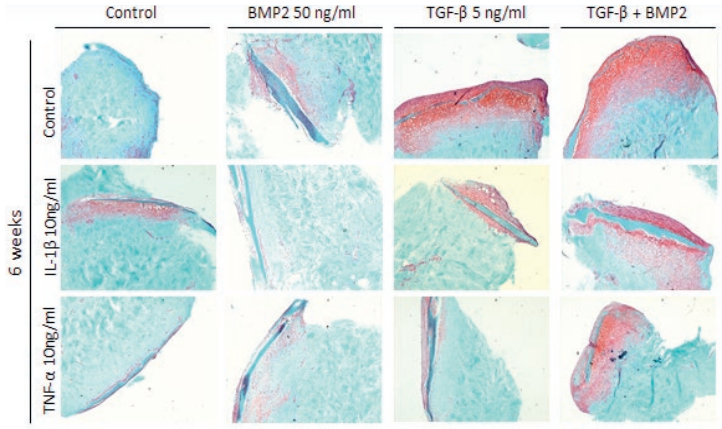


Figure 1 *Histology of periosteal explants.*

A: example to interpret histological sections. Chondrocytes (arrows), a cambium layer (C) and fibrous tissue (F) are shown (magnification 50x to 200x).
B: periosteal explants cultured for 4 and 6 weeks, stimulated with BMP2 and/or TGF-β, combined with exposure to IL-1β or TNF-α. Sections were stained with Safranin O and Fast Green (magnification 50x).

Results

The effect of growth factor induced osteophyte formation
BMP2 was not capable of inducing osteophyte formation. We observed proliferation of fibrous cells, but no cartilage-like cells were seen. On the other hand, TGF-β was capable of inducing osteophyte formation. Cartilage-like structures were observed after 4 weeks which were enlarged by 6 weeks. The combination of TGF-β and BMP2 was also capable of inducing osteophyte formation in periosteal explants.

After 4 weeks we observed more chondrogenesis in the culture stimulated with TGF-β combined with BMP2, compared to TGF-β alone. After 6 weeks the osteophytes were enlarged in the culture of combined stimulation with TGF-β and BMP2 (figure 1b).

Periosteal explants which were exposed to IL-1β alone, formed cartilage-like cells after 6 weeks, but hardly any cartilage matrix was seen. Furthermore, IL-1β inhibited cell proliferation in BMP2 stimulated explants. IL-1β completely blocked TGF-β induced osteophyte formation at 4 and 6 weeks. Strikingly, despite the block of osteophyte formation we did observe a fibrous layer that had formed on the explants after 6 weeks of TGF-β and IL-1β stimulation. When we combined TGF-β with BMP2, IL-1β inhibited osteophyte formation completely, but the thick fibrous layer was also present on the explants after 6 weeks similar to the layer observed when TGF-β alone was combined with IL-1β. This fibrous layer was not seen after 4 weeks on the explants (figure 1b).

We observed less cell proliferation after 4 weeks when we exposed BMP2 stimulated explants to TNF-α compared to BMP2 stimulation alone. After 6 weeks, TNF-α did not inhibit BMP2 induced proliferation. TNF-α was able to prevent osteophyte formation including the fibrous layer completely at both time points in TGF-β stimulated explants. However, TNF-α was not capable of inhibiting TGF-β plus BMP2 induced osteophyte formation after 4 weeks. After 6 weeks TNF-α was able to reduce TGF-β plus BMP2 induced osteophyte formation, but not completely (figure 1b).

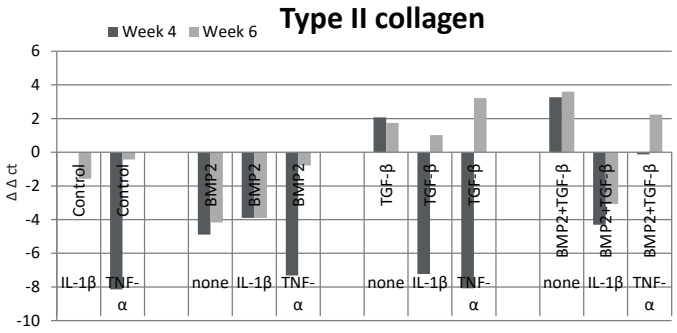


Figure 2 *Relative mRNA levels expression of type II collagen (Col2). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

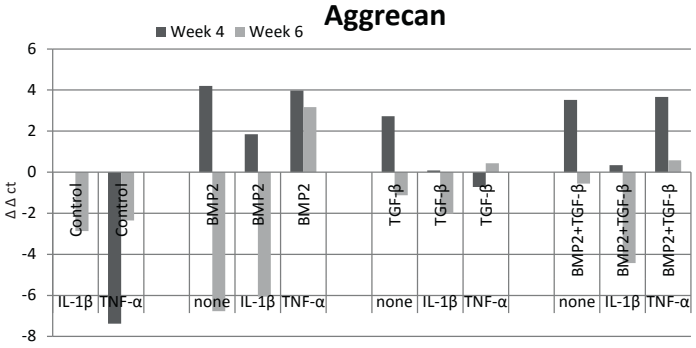


Figure 3 *Relative mRNA levels expression of aggrecan. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

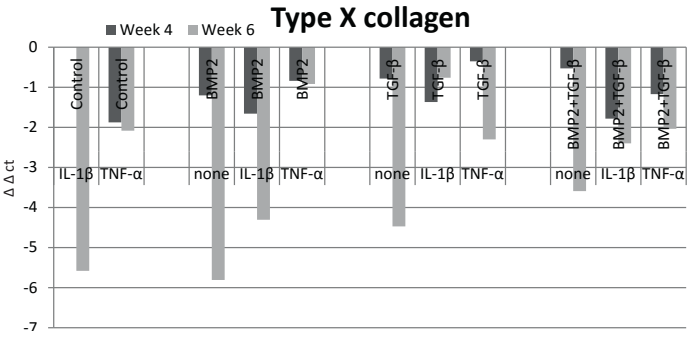


Figure 4 *Relative mRNA levels expression of type X collagen (Col10). CT values were first corrected for GAPDH, and then corrected for the unstimulated control*

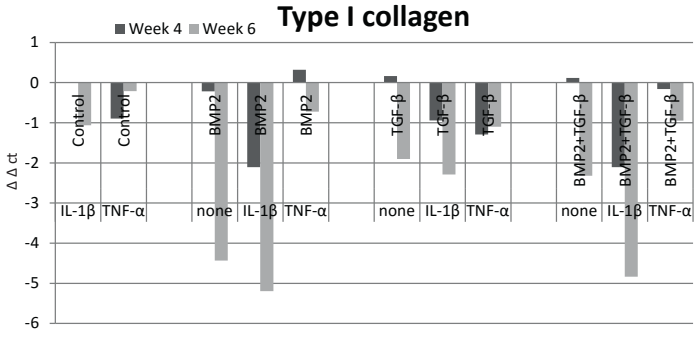


Figure 5 *Relative mRNA levels expression of type I collagen (Col1). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

Taken together, only conditions containing TGF-β resulted in osteophyte formation. The addition of BMP2 to TGF-β stimulation even induced chondrogenesis more than TGF-β alone. IL-1β inhibited chondrogenesis in all explants, but did not block the formation of a fibrous layer on the explant. TNF-α only blocked osteophyte formation in TGF-β stimulated explants. When we combined TGF-β with BMP2, TNF-α was no longer able to prevent osteophyte formation.

Gene expression of periosteal explants

Col2 mRNA expression

The gene expression of extracellular matrix molecule Col2 was decreased by BMP2 stimulation. TGF-β alone and when combined with BMP2 increased Col2 mRNA expression. These findings were consistent with histology, since we observed chondrogenesis in TGF-β alone or combined with BMP2 stimulated explants and no chondrogenesis in BMP2 stimulated explants in histology (figure 2).

IL-1β had no effect on the expression of Col2 in BMP2 stimulated explants. When the explants were stimulated with TGF-β alone or combined with BMP2, IL-1β decreased Col2 expression after 4 weeks. The Col2 decrease of IL-1β in TGF-β stimulated explants was no longer present after 6 weeks, but Col2 was still decreased after 6 weeks when TGF-β was combined to BMP2. On histology, we also found that IL-1β inhibited chondrogenesis (figure 2).

TNF-α decreased Col2 expression after 4 weeks in BMP2 stimulated explants, while there was an increase after 6 weeks. TNF-α down-regulated Col2 expression in TGF-β stimulated explants after 4 weeks and up-regulated Col2 after 6 weeks. TNF-α decreased Col2 expression after 4 and 6 weeks both when the explants are combined stimulated with TGF-β and BMP2 (figure 2).

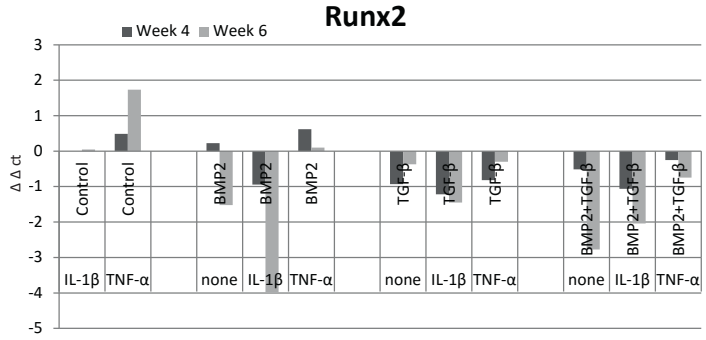


Figure 6 *Relative mRNA levels expression of runt-related transcription factor-2 (Runx2). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

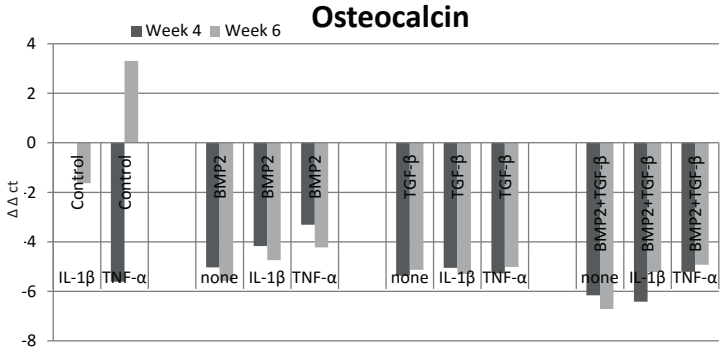


Figure 7 *Relative mRNA levels expression of osteocalcin. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

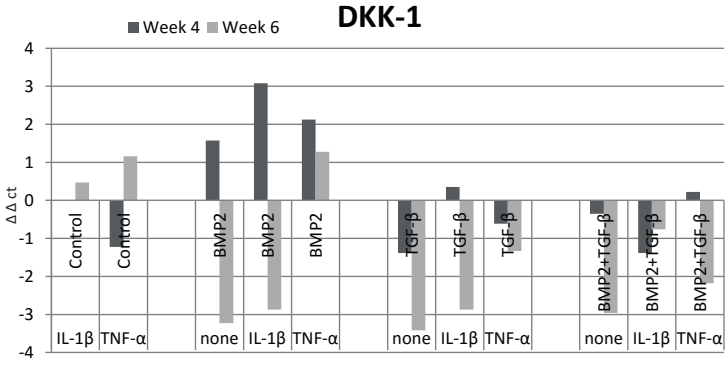


Figure 8 *Relative mRNA levels expression of DKK-1. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

Aggrecan mRNA expression

The expression of aggrecan was up-regulated in all growth factor groups after 4 weeks and dropped after 6 weeks. This aggrecan drop is also seen in chondrogenesis of embryonic stem cells after 30 days [23] and 10 weeks [24].When we exposed the explants to IL-1β, aggrecan expression was down-regulated in all groups after 4 and 6 weeks. TNF-α did not inhibit aggrecan expression in BMP2 or TGF-β plus BMP2 stimulated explants. TNF-α had no obvious effect on aggrecan expression of TGF-β stimulated explants (figure 3).

Col10, Col1, Runx2 and osteocalcin mRNA expression

Gene expression of the hypertrophic chondrocyte stage marker (Col10) and osteogenesis markers (Col1, Runx2 and osteocalcin) was down

regulated in aproximately all periosteal explants. IL-1β and TNF-α administration blocked the inhibition of chondrocyte hypertrophy (Col10) when the explants were stimulated with BMP, TGF-β or combined after 6 weeks (fig. 4). The different conditions downregulated or barely had any effect on Col1 (fig. 5), Runx2 (fig. 6) and osteocalcin (fig. 7) expression. This downregulation indicates that bone formation was not induced. This is consistent with the histology findings, where no bone was seen.

DKK-1 mRNA expression

The proposed osteophyte inhibitor DKK-1 was downregulated by TGF-β and TGF-β plus BMP2. When these explants were exposed to IL-1β, DKK-1 expression was upregulated in all conditions, except for the explants stimulated with BMP2 plus TGF-β after 4 weeks. TNF-α, also partially blocked the down-regulation of DKK-1 in all groups (figure 8).

Discussion

In this study, we induced bone formation in bovine derived periosteal cells by adding different mediators that are essential to the development of osteophytes. Our primary goal was to identify whether the absence of osteophytes in RA is due to the presence of the inflammatory we wanted to identify if cytokine TNF-α via the potent osteophyte inhibitor Dickkopf-1. Secondarily, we wanted to determine the potential inhibitory effect of IL-1β and TNF-α on growth factor induced osteophyte formation.

In scientific literature, BMP2 has been described as an important factor in chondrogenic differentiation and cartilage formation during EBF [25]. BMP2 increases Col2 and aggrecan expression and induces formation of hypertrophic chondrocytes in rat periosteum-derived cells during in vitro chondrogenesis in an aggregate culture after 14 days [25]. We found that BMP2 is not capable of the induction of chondrogenesis or osteophyte formation in bovine explant periosteum, considering histology and Q-PCR results. Our Q-PCR results revealed that BMP2 down-regulated chondrogenesis-marker Col2 in our experiments. The effect of IL-1β and TNF-α on osteophyte formation could not be determined, since no osteophytes were formed in BMP2 stimulated explants.

In previous rabbit experiments, TGF-β stimulates chondrogenesis of periosteal explants cultured in vitro [14, 26]. Furthermore, TGF-β has the ability to induce Col2 expression by mesenchymal cells [27]. In our research, we observed that TGF-β alone, or when combined to BMP2 was able to induce chondrogenesis. TGF-β or TGF-β plus BMP2 induced Col2 and aggrecan expression. BMP2 alone could not induce chondrogenesis, but when combined to TGF-β chondrogenesis was enhanced, which can most likely be attributed to the fact that BMP2 is more important in the later phases of this process [8]. Thus, TGF-β alone was able to induce chondrogenesis and this effect was enhanced by BMP2.

IL-1β is a potent inhibitor of Col2 and proteoglycans synthesis [12], which are essential to the chondrogenic phase of osteophyte formation. IL-1β has a suppressive effect on chondrocyte proteoglycan synthesis and it also stimulates the chondrocyte to release destructive proteases, which mediate the breakdown of cartilage [4].

Unlike IL-1β as a destructive mediator in both OA and RA [4], cartilage-like cells without cartilage matrix were seen after 6 weeks of stimulation in our experiments. We observed that IL-1β inhibited Col2 and aggrecan expression. Explants stimulated with TGF-β alone or combined to BMP2, showed less chondrogenesis when they were exposed to IL-1β. These results were consistent with literature that IL-1β inhibits chondrogenesis [4, 12]. We also observed that explants exposed to IL-1β had a fibrous layer. IL-1β inhibited chondrogenesis, but did not block the production of the fibrous layer on the explant that is also observed in osteophyte development. Thus IL-1β only fully blocks the chondrogenic/osteogenic part of osteophyte formation.

TNF-α is a potential osteophyte inhibitor by inducing the breakdown of extracellular matrix molecules of articular cartilage [11]. Moreover, TNF-α is suggested to have a negative effect on osteophyte formation via DKK-1 [5]. In earlier mouse experiments, we found that osteophyte formation did not increase during inflammatory arthritis in TNF-α knockout mice [13]. Furthermore, we observed osteophytes are formed through IBF in RA models [2], whereas in our model, IBF was not induced, but we induced chondrogenesis instead. Therefore, we could not determine the effect of TNF-α in RA on osteophyte formation. The effect of TNF-α on Col2 expression in BMP2 stimulated explants is irrelevant, since BMP2 was not able to induce chondrogenesis. TNF-α blocked osteophyte formation and down-regulated Col2 and aggrecan expression in TGF-β stimulated explants. However, TNF-α did not fully block osteophyte formation completely in TGF-β plus BMP2 stimulated explants. The addition of BMP2 to TGF-β clearly overruled the inhibitory effect of TNF-α. TNF-α down-regulated aggrecan expression in TGF-β only stimulated explants, but when TGF-β was combined with BMP2, TNF-α was not longer able to down-regulate aggrecan expression. TNF-α did not upregulate DKK-1 expression remarkably. We found that TNF-α was able to inhibit TGF-β induced chondrogenesis. The addition of BMP2 to TGF-β stimulation overruled the effect of TNF-α.

Conclusion

TNF-α may block osteophyte formation, but only in TGF-β stimulated explants and not when TGF-β was combined with BMP2. Since only marginal effects were found on RNA levels, we could not confirm a possible blocking effect of TNF-α on osteophyte formation. We have found that IL-1β was a potent osteophyte inhibitor by blocking the chondrogenic/osteogenic part of osteophyte formation.

Osteophytes are present in OA an absent in RA. It is suggested that osteophytes are absent in RA due to the presence of TNF-α . We did not see chondrogenesis in BMP-2 stimulated explants. No chondrogenesis was observed when TGF-β stimulated explants were exposed to TNF-α, but chondrogenesis was observed when TGF-β plus BMP2 stimulated explants were exposed to TNF-α. It remains unclear whether TNF-α blocks osteophyte formation in RA via DKK-1 since no significant effects of TNF-α on the expression of DKK1 were found.

Considering these findings, TNF-α may have an inhibiting effect on osteophyte formation, but most likely not via DKK-1. To determine via which signalling pathway TNF-α blocks osteophyte formation in RA, further research is needed.

References

1. Kuettner KE, Cole AA: Cartilage degeneration in different human joints. OsteoarthritisCartilage 2005, 13(2):93-103.
2. Cohen MM, Jr.: The new bone biology: pathologic, molecular, and clinical correlates. AmJMedGenetA 2006, 140(23):2646-2706.
3. Pelletier JP, Martel-Pelletier J, Abramson SB: Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis and rheumatism 2001, 44(6):1237-1247.
4. van den Berg WB: The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. ZRheumatol 1999, 58(3):136-141.
5. Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, Korb A, Smolen J, Hoffmann M, Scheinecker C et al: Dickkopf-1 is a mas-

ter regulator of joint remodeling. NatMed 2007, 13(2):156-163.
6. van der Kraan PM, van den Berg WB: Osteophytes: relevance and biology. OsteoarthritisCartilage 2007, 15(3):237-244.
7. Yusuf E, Kortekaas MC, Watt I, Huizinga TW, Kloppenburg M: Do knee abnormalities visualised on MRI explain knee pain in knee osteoarthritis? A systematic review. AnnRheumDis 2011, 70(1):60-67.
8. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FA, van den Berg WB, van der Kraan PM: Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum 2007, 56(12):4065-4073.
9. Sanyal A, Oursler MJ, Clemens VR, Fukumoto T, Fitzsimmons JS, O'Driscoll SW: Temporal expression patterns of BMP receptors and collagen II (B) during periosteal chondrogenesis. JOrthopRes 2002, 20(1):58-65.
10. Scharstuhl A, Glansbeek HL, van Beuningen HM, Vitters EL, van der Kraan PM, van den Berg WB: Inhibition of endogenous TGF-beta during experimental osteoarthritis prevents osteophyte formation and impairs cartilage repair. JImmunol 2002, 169(1):507-514.
11. Wehling N, Palmer GD, Pilapil C, Liu F, Wells JW, Muller PE, Evans CH, Porter RM: Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways. Arthritis and rheumatism 2009, 60(3):801-812.
12. Lotz M, Rosen F, McCabe G, Quach J, Blanco F, Dudler J, Solan J, Goding J, Seegmiller JE, Terkeltaub R: Interleukin 1 beta suppresses transforming growth factor-induced inorganic pyrophosphate (PPi) production and expression of the PPi-generating enzyme PC-1 in human chondrocytes. ProcNatlAcadSciUSA 1995, 92(22):10364-10368.
13. Mutualatupauw J, Blaney Davidson EN, van der Kraan PM, Koenders M: Osteophyte formation during murine osteoarthritis and inflammatory arthritis. unpublished data 2010.
14. O'Driscoll SW, Recklies AD, Poole AR: Chondrogenesis in periosteal explants. An organ culture model for in vitro study. JBone Joint SurgAm 1994, 76(7):1042-1051.
15. Wang F, Ying Z, Duan X, Tan H, Yang B, Guo L, Chen G, Dai G, Ma Z, Yang L: Histomorphometric analysis of adult articular calcified cartilage zone. Journal of structural biology 2009, 168(3):359-365.
16. Jay GD, Fleming BC, Watkins BA, McHugh KA, Anderson SC, Zhang LX, Teeple E, Waller KA, Elsaid KA: Prevention of cartilage degeneration and restoration of chondroprotection by lubricin tribosupplementation in the rat following anterior cruciate ligament transection. Arthritis and rheumatism 2010, 62(8):2382-2391.
17. Sarropoulou E, Nousdili D, Kotoulas G, Magoulas A: Functional Divergences of GAPDH Isoforms During Early Development in Two Perciform Fish Species. MarBiotechnol(NY) 2011.
18. Velleman SG: The role of the extracellular matrix in skeletal development. PoultSci 2000, 79(7):985-989.
19. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB: Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. OsteoarthritisCartilage 1998, 6(5):306-317.
20. Kuznetsov SA, Cherman N, Robey PG: In vivo bone formation by progeny of human embryonic stem cells. Stem Cells Dev 2011, 20(2):269-287.
21. Liu JC, Lengner CJ, Gaur T, Lou Y, Hussain S, Jones MD, Borodic B, Colby J, Steinman HA, van Wijnen AJ et al: RUNX2 expression utilizes the RUNX2P1 promoter to establish osteoprogenitor cell number for normal bone formation. JBiolChem 2011.
22. Estevao MD, Silva N, Redruello B, Costa R, Gregorio S, Canario AV, Power DM: Cellular morphology and markers of cartilage and bone in the marine teleost Sparus auratus. Cell Tissue Res 2011, 343(3):619-635.
23. Yamashita A, Nishikawa S, Rancourt DE: Identification of five developmental processes during chondrogenic differentiation of embryonic stem cells. PLoSOne 2010, 5(6):e10998.
24. Lin L, Shen Q, Xue T, Yu C: Heterotopic ossification induced by Achilles tenotomy via endochondral bone formation: expression of bone and cartilage related genes. Bone 2010, 46(2):425-431.
25. Hanada K, Solchaga LA, Caplan AI, Hering TM, Goldberg VM, Yoo JU, Johnstone B: BMP-2 induction and TGF-beta 1 modulation of rat periosteal cell chondrogenesis. Journal of cellular biochemistry 2001, 81(2):284-294.
26. Mizuta H, Sanyal A, Fukumoto T, Fitzsimmons JS, Matsui N, Bolander ME, Oursler MJ, O'Driscoll SW: The spatiotemporal expression of TGF-beta1 and its receptors during periosteal chondrogenesis in vitro. JOrthopRes 2002, 20(3):562-574.
27. Stevens MM, Marini RP, Martin I, Langer R, Prasad Shastri V: FGF-2 enhances TGF-beta1-induced periosteal chondrogenesis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2004, 22(5):1114-1119.

CORRECT ANSWERS TO THE EXAM QUESTIONS

Question 1: Natural killer cells (Answer B, 67% answered correctly)
Natural killers cells are a type of cytotoxic lymphocyte. They are a part of the innate immune system and can be found in the bone marrow, spleen, blood and liver. NK cells are known for their rapid response and direct targeting of cancer and virus-infected cells. In addition they are the main producers of interferon-gamma, which among other things, activates macrophages.

Question 2: The cost of health care (Answer C, 42% answered correctly)
According to the recent announcement of next year's national budget, the Netherlands will spend 74.6 billion euros on health care in the coming year. This is the equivalent of about 200 million euros a day.

MISPLACED INTRAUTERINE DEVICE

Anna M. van Boekel

A 29 year old woman, gravida 3, para 2, was referred to the Gynaecology department because the removal strings of her intrauterine device (IUD) had not been visible during follow-up. The IUD had been inserted 6 weeks earlier without any complications. A transvaginal ultrasound did not show any signs of the IUD being in utero. An abdominal X-ray was performed (Figure 1) which showed the IUD appeared to be located in the right upper quadrant of the abdomen. The patient was scheduled for laparoscopic removal. During the procedure the IUD was found to be embedded in the greater omentum and removed without any further complications. Afterwards a new IUD was vaginally inserted under laparoscopic guidance.

The IUD is a commonly used long-acting and reversible contraceptive device. Six weeks after the insertion of an IUD, patients will usually visit their general practitioner or gynaecologist to determine whether the removal strings are still visible. Visualisation of the strings in the external orifice indicates that the IUD is in place. However, in 4.5% to 18.0% of patients the removal strings cannot be seen [1]. Most often this is due to string retraction into the cervical canal or uterine cavity, however it can also occur in expulsion of the IUD or uterine perforation.

Most uterine perforations occur during IUD insertion [2]. Perforations can be partial, in which a part of the device is still in utero, or complete, in which the entire device has moved into the abdominal cavity [3]. The displaced IUD might lead to damage of the visceral organs, predominantly the intestines, such as fibrosis, perforation, bowel obstruction, mesenteric perforation, bowel infarction, rectal strictures and rectouterine fistula [3]. The frequency of uterine perforation is estimated to be 0.05 to 13 per 1000 insertions[3]. The risk of uterine perforation is higher in copper IUDs, in insertions shortly after pregnancy, in breastfeeding women and in patients with a retroverted uterus [2–4].

Our patient had received the IUD a few months after delivering her second child, while she was still breastfeeding. The Dutch College of General Practitioners strongly advises against placing an IUD within 4 to 6 weeks after pregnancy [5]. However even at insertion 6 months after pregnancy the risk of perforation is still increased [2]. Possible symptoms of perforation include abdominal pain and abnormal vaginal bleeding but many patients, such as our patient, are asymptomatic [3, 5]. Therefore, further clinical evaluation is indicated when the strings of the IUD are not visible during the follow-up visit. The first step in evaluation is a transvaginal ultrasound to determine whether the device is visible in the uterine cavity, followed by radiographic evaluation of the abdomen and pelvis when the IUD is not localised in the uterine cavity [3, 4].

Treatment of asymptomatic patients with a perforated IUD is controversial. The risks of surgical removal may be greater than the risk of the misplaced IUD. Nonetheless, the WHO recommends that displaced IUDs should always be removed to prevent possible complications that can occur due to migration and adhesion formation [3].

Conclusion

Uterine perforation and subsequent migration into the abdominal cavity is a rare but not uncommon complication of IUDs. Clinicians should be aware of this complication when the removal strings cannot be seen during follow-up. Extra vigilance is required in postpartum or breastfeeding patients. Further evaluation is necessary to exclude a uterus perforation.

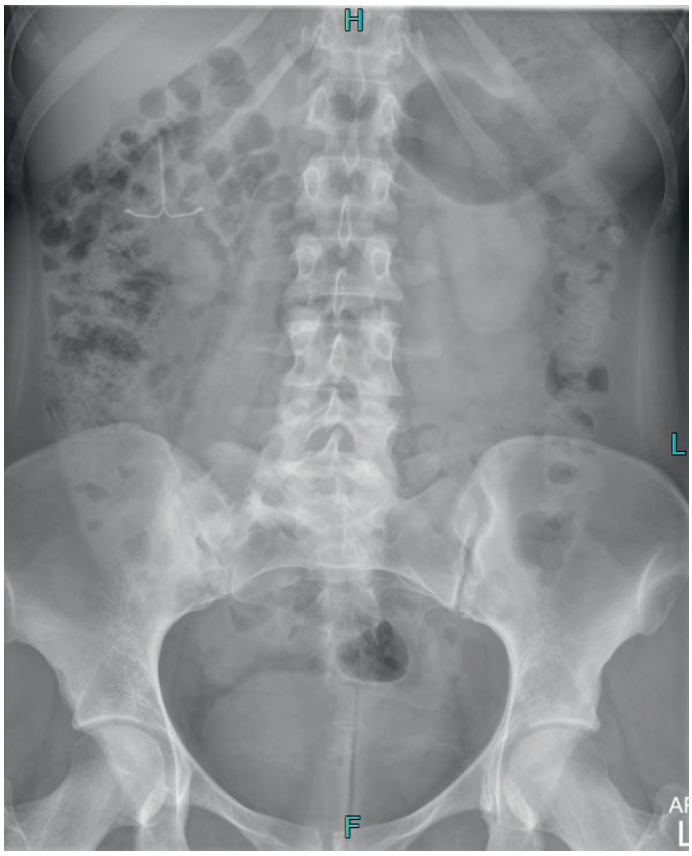


Figure 1 *Abdominal X-ray showing the IUD in the upper abdomen.*

References

1. Marchi NM, Castro S, Hidalgo MM, Hidalgo C, Monteiro-Dantas C, Villarroeal M, Bahamondes L: Management of missing strings in users of intrauterine contraceptives. Contraception 2012, 86:354–358.
2. Kaislasuo J, Suhonen S, Gissler M, Lähteenmäki P, Heikinheimo O: Intrauterine contraception: incidence and factors associated with uterine perforation--a population-based study. Hum Reprod 2012, 27:2658–63.
3. Rahnemai-Azar A a., Apfel T, Naghshizadian R, Cosgrove JM, Farkas DT: Laparoscopic Removal of Migrated Intrauterine Device Embedded in Intestine. JSLS J Soc Laparoendosc Surg 2014, 18:e2014.00122.
4. Alderlieste WC, Jacobs JJWM: [No visible strings during an IUD check-up]. Ned Tijdschr Geneeskd 2015, 159:A8413.
5. Anticonceptie | NHG [https://www.nhg.org/standaarden/samenvatting/anticonceptie]

LINKING THE PHARMACOKINETICS OF SULFADIMIDINE TO N-ACETYLTRANSFERASE 2 (NAT2) GENE POLYMORPHISMS IN BIOMEDICAL SCIENCES STUDENTS; A PHENOTYPE-GENOTYPE CORRELATION

Lars A.A. Damen[1], Thom K. van der Made[1]

Corresponding author: Lars A.A. Damen (lars.damen@student.ru.nl)

[1] Master Student Biomedical Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands

ABSTRACT:

INTRODUCTION: Prediction of clinical effects after drug administration is of paramount importance for the improvement of beneficial effects and minimization of adverse drug reactions in daily practice. Clinical effects can differ between individuals as a result of variation in expression of metabolizing enzymes. The NAT2 protein is a metabolizing enzyme responsible for bioactivation and detoxification of commonly prescribed drugs such as isoniazid, which is used to treat tuberculosis. This protein is encoded by the NAT2 gene, a gene which is highly polymorphic. Two polymorphisms common in the Caucasian population are studied, NAT2*5B and NAT2*7. We expect that NAT2*7 leads to a slow acetylating status, whereas NAT2*5B does not.
OBJECTIVE: The aim of this study was to link NAT2 activity to a specific genotype pattern.
METHODS: 9 Students of Biomedical Sciences at the Radboud University ingested a 1500 mg dose of sulfadimidine. Sulfadimidine concentrations were measured in a blood sample and several saliva and urine samples were obtained from the students to determine their metabolism phenotype. Genotype was determined by restriction fragment length polymorphism using the bamH1 and kpn1 restriction enzymes.
RESULTS: Of the 9 students, 7 showed a slow phenotype with a half-life of 10.2 hours (SD=6.61 hours) and a metabolic rate of 45% (SD=6.61%). Two of the slow phenotype students had a mutation in both alleles, one showed a mutation in NAT2*7, 2 students in NAT2*5B and one student had no mutations. The 2 students with a fast acetylating phenotype had a half-life of 3.0 hours (SD = 2.47 hours) and a metabolic rate of 83% (SD = 14.7%). These students had either a mutation in NAT2*5B or NAT2*7.
CONCLUSION: There might not be a relation between the genotype of two common polymorphisms in the NAT2 gene and acetylating phenotype, although the small sample size limits our ability to draw firm conclusions.

WHAT'S KNOWN: There is evidence that phenotypic activity correlates to the NAT2 genotype. Different ethnic populations, with a different genetic make-up, show a different phenotypic status.

WHAT'S NEW: In this study, we specifically used a cohort of Biomedical students to investigate the relationship between NAT2 phenotype and genotype, because differences in lifestyle pattern may influence metabolic rate. However, due to the small sample size, no definitive conclusion could be drawn.

KEYWORDS: NAT2, polymorphisms, genetic association studies, pharmacokinetics

Introduction

Prediction of the clinical effects after drug administration is of paramount importance for the improvement of beneficial effects and minimization of adverse drug reactions in daily clinical practice. However, this is difficult due to large inter-individual variation in drug-responses. Genetic heterogeneity in enzymes responsible for metabolism and transport of drugs, drug targets and disease etiology, but also environmental factors, may determine the response to drugs. In most cases biomarkers or genetic screening are used to improve clinical practice. As for genetic screening, Single Nucleotide Polymorphisms (SNPs) are used to determine which genetic profile a patient expresses and whether this profile can result in the desired response to a particular drug.

This 'personalized medicine' approach could lead to avoidance of unwanted side effects or drug plasma concentrations outside of the therapeutic window. Besides the advantages at an individual level, prediction of the clinical effects will also reduce health care costs. Davies et al. showed that adverse drug reactions (ADR) account for one fifth of hospital readmissions in the UK [11]. A study conducted in France indicated an incidence of 3.6 % of hospital admissions due to ADRs [12].

Genetic screening of the Arylamine N-acetyltransferase (NAT2) gene may provide important information about the response to commonly prescribed drugs such as isoniazid, hydralazine, and sulfamethoxazole [1]. Isoniazid, for instance, is a common first-line anti-tuberculosis drug. If the concentrations of this drug become too high, isoniazid has the potential to induce liver damage [18]. Isoniazid is metabolized by the genetically polymorphic arylamine N-acetyltransferase type 2 (NAT2). An increased activity of NAT2 is related to increased acetylation capacity for drugs that are metabolized by the NAT2 enzyme.

The NAT2 gene encodes the NAT2 protein, which is a phase II metabolizing enzyme responsible for metabolism by N-acetylation or O-acetylation of drug compounds containing aromatic amines and hydrazines [1]. In effect, this enzyme catalyzes the bioactivation and/or detoxification of drugs as isoniazid, hydralazine, and

sulfamethoxazole [1].

NAT2 is a single, intronless gene, which is located at 8p22 [1]. It is considered to be highly polymorphic, composing seven general nucleotide changes that are associated with its acetylating phenotype [1]. 191 G > A, 341 T > C, 590 G > A, and 857 G > A, which reduce the acetylation activity of NAT2; and 282 C > T, 481 C > T, and 803 A > G, which did not [1]. This study will focus on the NAT2*5B (C481T) and NAT2*7 (G857A) polymorphisms, because these polymorphisms are not yet evaluated in students, whereas these are common polymorphisms in the Caucasian population. NAT2*4 is considered to be the wildtype allele.

Sulfadimidine is one of the drugs that is metabolized by the NAT2 protein [16]. Sulfadimidine is an antibacterial agent, which has been used to treat urinary tract infections since 1942. Nowadays this compound is rarely used in the clinic, because there are more effective compounds on the market to treat urinary tract infections. However, this drug offers a great possibility to study the acetylator phenotype in humans. This drug is not harmful when given once in an amount of 1500 mg. Sulfadimidine is predominately metabolized to N4-acetylsulfadimidine [16]. In this paper, we will further distinguish between rapid and slow acetylators. In the literature, participants were classified as slow or fast acetylators based on the acetylated / unchanged sulfadimidine plasma level ratio, using a threshold value of 70% [1]. The prevalence of slow and fast acetylators differs between ethnic populations [1,4-6].

The aim of this study was to link NAT2 activity to a specific genotype pattern. NAT2*7 leads to a slower acetylating status, whereas NAT2*5B will not [1]. Therefore we expect that students without mutations, so called wild types, are fast acetylators. One 'slow' allele will lead to a slow acetylation phenotype, those with two mutations in NAT2*7'alleles will show a more extreme phenotype [19]. We expect students with SNPs in NAT2*5B not to have a slower acetylating status. We used PCR to determine the genotype of the students. Urine and saliva samples were analyzed at specific time points to produce time-concentration curves and to calculate pharmacokinetic parameters, which include the metabolic ratio, metabolic clearance, half-life and the Area Under the Curve (AUC).

Methods

Study design

Biomedical students enrolled in the course Drugs Development at the Radboud University were included in our study on a voluntary basis. This population consisted of seven Caucasian and two non-Caucasian students. Students were excluded in case of liver or kidney disorders, pregnancy, G6PDH-deficiency or after prior use of sulfa-compounds. First, the participants gave their informed consent after being informed by a physician, due to admission of medication and extraction of one blood sample and several saliva and urine samples. Because participants were required to be sober at the start of the experiment, they were only allowed to drink water in the 9 hours prior to the start of experiment. Half an hour before administering sulfadimidine, saliva and urine samples were collected for baseline measurements. Subsequently, all students ingested 1500mg of sulfadimidine. During the 24 hours after administration of the drug, urine and saliva samples were collected at specific time points (table 1). These samples were subsequently analyzed for sulfadimidine and the N-acetylsulfadimidne in plasma and urine, while saliva samples were only analyzed for sulfadimidine. Four and half hours after administration of the drug a blood sample (approximately 6 mL) was collected. This sample was analyzed for bound and unbound sulfadimidine and N-acetylsulfadimidine concentrations and for the presence of polymorphisms in NAT2*5B or NAT2*7 alleles.

DNA extraction

DNA was isolated from 1 ml peripheral blood using the Puregene DNA Isolation kit. First, 200 µl whole blood was added to a microtube containing 600 µl RBC lysis solution. The solution was vortexed and the supernatant removed. After that, 200 µl of a cell lysis solution was added. A protein precipitation solution (PP4) was used to precipitate the proteins. Subsequently 200 µl 100% isopropanol (IP5) and 200 µl 70% ethanol (ET6) were added, respectively, to precipitate DNA. Lastly, 50 µl DNA Hydration Solution (DH7) was added for DNA hydration.

PCR-sequencing

The primers were designed to amplify the human NAT2 gene sequence from position 761 bp to 1861 bp. The length of the whole PCR product was 1101 bp, which contained the NAT2*5B and NAT2*7 polymorphisms at bp 481 and bp 857 respectively in this fragment The primer sequences were NAT2-For2 5'-aactctaggaacaaattggac-3'and NAT2-Rev2 5'-tttctagcatgaatcactctg-3'. PCR reactions were performed with 1 µl DNA and 49 µl of the PCR mix. This mix consisted of 1.0 µl 10 µM NAT2-For2 primer, 1.0 µl 10 µM NAT2-Rev2 primer, 1.0 µl2.5 mM dNTP's, 5.0 µl 10x PCR buffer (Invitrogen), 2.0 µl 50 mM MgCl2 (Invitrogen), 38.75 µl mQ and 0.25 µl Taq DNA polymerase (Invitrogen). Besides the DNA samples we took mQ as negative control. The amplifications were done using the following PCR program. Initial denaturation was done at 94 ^{0}C for 5 minutes (step 1), another denaturation step at 94 ^{0}C for 30 s (step 2), 30 s at 58 ^{0}C (annealing, step 3), 1.5 minute at 72 ^{0}C (elongation, step 4). Repeat step [2-4] 39 times, followed by a step at 72 ^{0}C of 5 minutes. Amplification was verified by gel electrophoresis (1.5% agarose gel, at 125 Volt).

Digestion of PCR products with restriction enzymes

The restriction enzymes Kpn1 and BamH1 were used to determine mutations in the NAT2*5B and NAT2*7 gene sequence, respectively. Kpn1 cuts between G'GATCC and BamH1 cuts between GGTAC'C. First the mixes were made containing 1.0 µl 10x NEB buffer 3, 0.1 µl 100x BSA, 3.4 µl mQ and 0.5 µl BamH1 or Kpn1 restriction enzyme. Subsequently, 5 µl PCR product and 5 µl of the corresponding mix were added. The samples were incubated at 37 ^{0}C for 2 hours to digest the PCR product. A gel, containing 1.5 % agarose, was run for 15-20 minutes.

Determination of acetylator phenotype

Concentrations of sulfadimidine and N-acetylsulfadimidine in plasma, urine and saliva

Plasma, urine and saliva concentrations were measured according to standard procedures. (supplementary 1)

Free protein concentration in the blood

For determination of the free protein plasma concentration Amicon ultra-filtration membranes from the Centricon system were used. These filters, with a cut-off of 30000 Dalton, allow small proteins like sulfadimidine to pass the filter membrane, whereas plasma proteins are not able to pass.

Renal Clearance
The renal clearance was calculated by dividing the total quantity of the drug excreted in the urine by the AUC.

Statistical analysis
Regression lines, standard deviations and t-tests were performed using graphpad prism 4.03 for Windows, GraphPad Software, San Diego California USA. Only an effect can be determined when the difference of a variable between groups is significantly different (P<0,05)

Results

After collecting urine, saliva and blood sample measurements, time-concentration curves were plotted using the measured concentrations of drug and metabolite. In our data set, differences between fast and slow metabolizers were observed in AUC, half-life, metabolic rate and plasma clearance. Figure 1 shows the concentration curves of a slow and a fast metabolizer. Calculated data from the subjects (n=9) and two historical data sets are depicted in table 2. Acetylating phenotype was based on the metabolic ratio <70% (NacS/ total dose ingested, as shown in the cumulative excretion- NacS column). In subject two, acetylating phenotype was not based on metabolic ratio but on half-life. The obtained values are summarized and shown in table 2. One of the factors that show whether someone is most likely a slow or fast metabolizer is the AUC. A high AUC means that the drug is present in the body for a longer period at a higher concentration. However, no significant difference can be observed in the AUC between both groups (784 µg.h/mL 95%CI -1835-3403) although subject 3 and 4 had a very high AUC. In these two subjects also a high half-life was observed. All slow acetylators showed a higher mean half-life, with a mean increase of 7,1 hours compared to the fast acetylators, although this difference is not significant (95%CI -7,5-21,7)

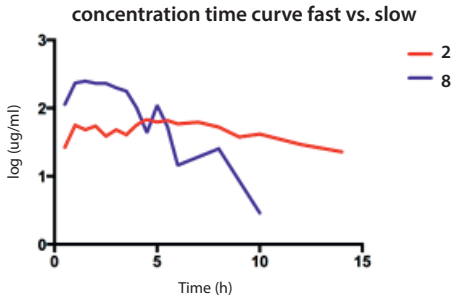


Figure 1 *Time-concentration curve of a fast and a slow acetylators. The log(concentration sulfadimidine) was plotted against the time. Student #2 is a slow metabolizer, this student has an higher AUC and t1/2. Student #8 is a fast metabolizer and shows a higher peak concentration, while the clearance of the drug is much faster.*

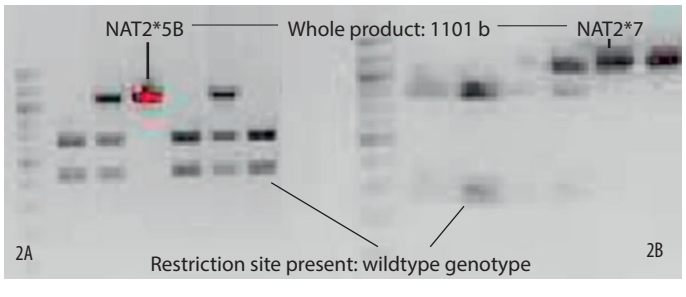


Figure 2*: Genotype patterns in fast and slow acetylators. Mutations in bamH1 (right), kpn1 (left) or both are depicted in the diagrams. Two wildtype alleles are present when one fragment at 1101 bp is visible, if there is one mutation present (heterozygous),3 bonds will be visible, two bonds will be visible if two mutations are present (homozygous).*

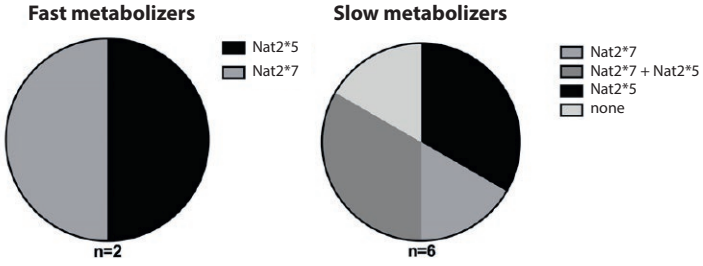


Figure 3 The genotype of fast and slow metabolizers. *The fast metabolizers have a mutation in the Nat2*5 or Nat2*7. Slow metabolizers may have a genotype which has no, one or two mutations.*

Plasma clearance and metabolic ratio were decreased in slow acetylators with 6,8 L/h (95% CI -3,3 ; 16,9) and 35,4% (95% CI 17,2 ; 53,6), respectively. Typical plasma concentration versus time curves for the two phenotypes are depicted in Figure 1, in which a higher AUC and an increase in half-life were observed for the slow acetylator. These subjects also showed differences in the amount of NacSulfadimidine and Sulfadimidine excreted in the urine, which appeared to be 50% and 30%, respectively, of the total amount of sulfadimidine ingested. Of the nine students included in the study, only eight were genotyped (two fast and six slow, based on metabolic ratio), due to refusal of one participant to extract a blood sample. Two separate alleles were analyzed for genotyping, NAT2*5B by kpn1 restriction and the NAT2*7 by BamH1 restriction. After restriction in the NAT2*5B allele, fragments should be 441bp and 660 bp in case the SNP is not present, while the fragment should be intact when the SNP is present (see figure 2A). A combination of both fragments and the whole PCR product indicates a heterozygous genotype. In the NAT2*7 allele the fragments should normally be found at 384bp and 817 bp, while presence of a SNP leads to an intact fragment (figure 2B).Two of the students who were genotyped had a mutation in both alleles, while one had no mutations. Three students had one mutation in NAT2*5B, two of them had a mutation in NAT2*7. These genotypes were coupled to the acetylating status of students based on metabolic ratio (table 1). The two fast metabolizers both had one mutation, but in a different allele. Of the six slow metabolizers, two had a mutation in both alleles, two had a mutation in NAT2*5B, one student had a mutation in the NAT2*7 and one had no mutations (figure 3).

Discussion

Variation in the activity of the NAT2 enzyme has important implications regarding therapeutic responses to commonly prescribed drugs such as isoniazid, hydralazine, and sulfamethoxazole. In order to predict different therapeutic responses between individuals, we performed a study to correlate phenotypic activity to NAT2 genotype in a cohort of Biomedical Sciences students. Our data suggest that there is no correlation between phenotypic activity and genotype.

A combination of polymorphisms in the NAT2*5B and NAT2*7 genes results in a slow acetylating phenotype. However, mutations in either NAT2*5B or NAT2*7 could not predict the phenotype. We expected that the NAT2*7 would lead to a slow phenotype based on the available literature, however, this mutation was also found in a fast acetylator [1]. Other study groups had already reported a good overall correlation between phenotypic activity and genotype [4,7,13]. In contrast, one study showed discordance between phenotype and genotype of 86% in a Hmong population in the USA [14]. The researchers concluded that environmental and genetic abnormalities may have contributed to the discordance.

In our study 22% of the students were fast acetylators, while 78% were slow. Literature showed that discrepancies between different studies regarding correlation between phenotype and genotype can be observed. Other studies already demonstrated concordance between phenotypic activity and genotype in different ethnic populations. In a study by Gross et al., 59.5% of the study population (American-Caucasian) was classified as slow acetylators [4]. Lucia Taja-Chayeba et al. showed that 59.8% of their population were slow acetylators [1]. Another study by Fuselli S. et al. demonstrated that 18%, 56% and 25% of their target population (Native Americans) were, respectively, fast, intermediate or slow acetylators [5]. Garcia Martin E. showed that inter-individual differences exist, for example individuals with Chinese descent are more often slow acetylators than in those with Japanese descent [6].

In this study, seven students were classified as slow acetylators, whereas two students were classified as fast acetylators. The mean half-life in slow and fast acetylators was 10.2 and 3.0 hours, respectively. This does correlate with existing literature, wherein the mean half-life of sulfadimidine in fast acetylators (n=6) was 1.70 +- 0.50 hours, the mean half-life in slow acetylators was 7.55 +- 0.90 hours [16]. The mean metabolic ratio in slow and fast acetylators was 45 and 83 %, respectively. In the literature also a difference in metabolic ratio was found between slow and fast acetylators, respectively 63 and 77% [16].

We have shown that there is no direct relation between phenotype and genotype. However, our study has several limitations. First, the number of persons participating in this study was low. Secondly, calculated 95% confidence intervals for the half-life were very large, these resulted in non-significant differences between the slow and fast acetylator groups. This may be due to practical errors. Thirdly, sulfadimidine plasma concentrations were calculated based on a saliva/plasma ratio, which was measured at one specific time point. This was less invasive for the students and it was assumed that this ratio could be extrapolated to all the other time points. Although this gives a relatively accurate concentration-time relation, the saliva/plasma ratio might not be the same over the whole period. At time of measurement concentrations of sulfadimidine can be altered due to the production of saliva, while the same amount of drug is still excreted. Production of saliva depends for example on food consumption, which we did not monitor.

Another point of discussion is that we studied polymorphisms in the NAT2*5B and NAT2*7 gene, whereas polymorphisms at other sites in the coding region or in the non-coding regulatory regions of NAT2 can cause a difference in the acetylating phenotype. Currently there are 61 alleles associated with the acetylating phenotype according to 'Consensus Human Arylamine N-Acetyltransferase Gene Nomenclature' [1]. However, it is unlikely that this has led to significant confounding, since the NAT2*5B and NAT2*7 polymorphisms are most common in the Caucasian population. Furthermore, gender was not evenly distributed in slow and fast acetylators, but we do not think that gender differences have a significant impact on acetylating activity. Also these results cannot be extrapolated to other ethnicities since interethnic difference can result in different outcomes. Finally, in some cases assumptions had to be made in order to obtain reliable graphs due to practical errors. In this situation, values representative for a healthy individual were used. In subject 2, the half-life of sulfadimidine was taken instead of the metabolic ratio to determine acetylating activity, because of too many missing values. The time-concentration curve of subject 2 was comparable to that of the other slow acetylators.

Recommendations

Further studies with a larger number of individuals are required to demonstrate the correlation between phenotype and genotype for NAT2 in students. To acquire more accurate data, these studies should be performed with whole genome screening instead of focusing on two polymorphisms. Also the use of a trimodal (slow/intermediate/fast) may be beneficial, because this model might represent the clinical situation better. Furthermore, other drugs than sulfadimidine may be used to give a better representation of the acetylation of drugs such as isoniazid. In conclusion, this study shows that there might not be a relation between the genotype of two common polymorphisms in the NAT2 gene and acetylating phenotype, although the small sample size limits our ability to draw conclusions.

Acknowledgements

We would like to thank Roos Masereeuw for her knowledge and supervision.

References

1. Taja-Chayeb L, Gonzalez-Fierro A, Miguez-Munoz C, Trejo-Becerril C, Cruz-Hernandez Ede L, Cantu D, et al. Acetylator status and N-acetyltransferase 2 gene polymorphisms; phenotype-genotype correlation with the sulfamethazine test. Pharmacogenetics and genomics. 2011;21(12):894-901. Epub 2011/09/29.
2. Chen B, Zhang WX, Cai WM. The influence of various genotypes on the metabolic activity of NAT2 in a Chinese population. European journal of clinical pharmacology. 2006;62(5):355-9. Epub 2006/03/30.
3. Selinski S, Blaszkewicz M, Ickstadt K, Hengstler JG, Golka K. Improvements in algorithms for phenotype inference: the NAT2 example. Current drug metabolism. 2014;15(2):233-49. Epub 2014/02/15.
4. Gross M, Kruisselbrink T, Anderson K, Lang N, McGovern P, Delongchamp R, et al. Distribution and concordance of N-acetyltransferase genotype and phenotype in an American population. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 1999;8(8):683-92. Epub 2000/04/01.
5. Fuselli S, Gilman RH, Chanock SJ, Bonatto SL, De Stefano G, Evans CA, et al. Analysis of nucleotide diversity of NAT2 coding region reveals homogeneity across Native American populations and high intra-population diversity. The pharmacogenomics journal. 2007;7(2):144-52. Epub 2006/07/19.
6. Garcia-Martin E. Interethnic and intraethnic variability of NAT2 single nucleotide polymorphisms. Current drug metabolism.

2008;9(6):487-97. Epub 2008/08/06.
7. Meisel P, Arndt D, Scheuch E, Klebingat KJ, Siegmund W. Prediction of metabolic activity from genotype: the gene-dose effect of N-acetyltransferase. Therapeutic drug monitoring. 2001;23(1):9-14. Epub 2001/02/24.
8. Fischer E. Renal excretion of sulphadimidine in normal and uraemic subjects. Lancet. 1972;2(7770):210-2. Epub 1972/07/29.
9. Ylitalo P, Auterinen L, Marttinen A, Koivula T. Acetylator phenotyping with sulphadimidine in patients receiving isoniazid. Int J Clin Pharmacol Res. 1984;4(2):141-4.
10. Drayer DE, Reidenberg MM. Clinical consequences of polymorphic acetylation of basic drugs. Clinical pharmacology and therapeutics. 1977;22(3):251-8. Epub 1977/09/01.
11. Davies EC, Green CF, Mottram DR, Rowe PH, Pirmohamed M. Emergency re-admissions to hospital due to adverse drug reactions within 1 year of the index admission. British journal of clinical pharmacology. 2010;70(5):749-55. Epub 2010/11/03.
12. Benard-Laribiere A, Miremont-Salame G, Perault-Pochat MC, Noize P, Haramburu F, the ESGobotFnopc. Incidence of hospital admissions due to adverse drug reactions in France: the EMIR study. Fundamental & clinical pharmacology. 2014. Epub 2014/07/06.
13. Rihs HP, John A, Scherenberg M, Seidel A, Bruning T. Concordance between the deduced acetylation status generated by high-speed: real-time PCR based NAT2 genotyping of seven single nucleotide polymorphisms and human NAT2 phenotypes determined by a caffeine assay. Clinica chimica acta; international journal of clinical chemistry. 2007;376(1-2):240-3. Epub 2006/10/03.
14. Straka RJ, Burkhardt RT, Lang NP, Hadsall KZ, Tsai MY. Discordance between N-acetyltransferase 2 phenotype and genotype in a population of Hmong subjects. Journal of clinical pharmacology. 2006;46(7):802-11. Epub 2006/07/01.
15. Djordjevic N, Carrillo JA, Ueda N, Gervasini G, Fukasawa T, Suda A, et al. N-Acetyltransferase-2 (NAT2) gene polymorphisms and enzyme activity in Serbs: unprecedented high prevalence of rapid acetylators in a White population. Journal of clinical pharmacology. 2011;51(7):994-1003. Epub 2010/08/31.
16. Vree TB, Hekster YA, Nouws JF, Baakman M. Pharmacokinetics, metabolism, and renal excretion of sulfadimidine and its N4-acetyl and hydroxy metabolites in humans. Therapeutic drug monitoring. 1986;8(4):434-9. Epub 1986/01/01.
17. Sim E, Abuhammad A, Ryan A. Arylamine N-acetyltransferases: from drug metabolism and pharmacogenetics to drug discovery. Br J Pharmacol. 2014;171(11):2705-25.
18. Huang YS. Recent progress in genetic variation and risk of antituberculosis drug-induced liver injury. J Chin Med Assoc. 2014;77(4):169-73..
19. Sim E, Abuhammad A, Ryan A. Arylamine N-acetyltransferases: from drug metabolism and pharmacogenetics to drug discovery. Br J Pharmacol. 2014;171(11):2705-25.
20. Singla N, Gupta D, Birbian N, Singh J. Association of NAT2, GST and CYP2E1 polymorphisms and anti-tuberculosis drug-induced hepatotoxicity. Tuberculosis (Edinb). 2014;94(3):293-8.
21. Winter HR, Unadkat JD. Identification of cytochrome P450 and arylamine N-acetyltransferase isoforms involved in sulfadiazine metabolism. Drug Metab Dispos. 2005;33(7):969-76.

THE 'VIERDAAGSE': 99 YEARS OF WALKING, 9 YEARS OF RESEARCH

Kim R.G. Cortenbach, Jasper M. Maters

Every year on the third Tuesday of July over 40,000 people show up at the start of the greatest walking event in the world: the 'Nijmeegse Vierdaagse' or Four Days Marches. People from all over the world and of all ages travel to Nijmegen to stretch their boundaries and hope to receive the well-known cross for the Vierdaagse. In 2015 42,684 hikers from over fifty countries participated. For four days, these participants walked 30, 40 or 50 kilometers each day. A few thousand military participants walked in uniform with a fully packed backpack. The goal of the event has always been to promote sport and exercise. Maria Hopman, a professor of integrative physiology at Radboudumc, is fascinated about this event and more in general about the relationship between exercise and health. In 2007 she set up a broad study in which participants were monitored and compared with non-participants. After a few years the study was expanded by including participants of two other running events, the 'Marikenloop' and 'Zevenheuvelenloop'. That is why we interviewed Maria Hopman about the study and her work as an avid researcher.

What was the main reason for starting the Vierdaagse Study?

It has all started because of the catastrophe in 2006. On the first day, two participants died, many were admitted to the hospital and hundreds were hospitalised. The Vierdaagse was cancelled and a committee was established. The committee advised the appointment of an exercise physiologist, for which I was asked. I was willing to do it but wanted to combine it with research because not much is known about the benefits and effects of hiking for 10-12 hours a day. The existing literature is only about the army, which consists mainly of young and healthy men. That is how we started in 2007 and this year was already the ninth edition.

Participants are asked to fill in a questionnaire. Could you give us more information about this questionnaire and the different events (Vierdaagse, Marikenloop, Zevenheuvelenloop).

Using the questionnaires we investigate the correlation between exercise and illness. The themes included are illness, health and exercise participation both in the past and in the present. This is the fifth year the questionnaires have been used and this year an additional theme, the food consumption, has been added. Our cohort, including the participants of the Zevenheuvelenloop, consists of 22,000 subjects. This is a huge number of subjects and therefore it is a great cohort in which we can investigate some interesting aspects of human physiology. Moreover, this kind of research will become more interesting over the course of the years, especially after twenty to thirty years. Then we will have some clear end points, which could give us some great insights.

> ***"The walker can consume a cup of coffee and start from our research centre, instead of waiting in the queue"***

Apart from the questionnaire, a thousand hikers are also invited to be tested at the Wedren (starting point of the Vierdaagse). Blood samples are taken and used to determine blood levels of minerals and vitamins. Also, fifty trained and fifty untrained hikers are intensively monitored with regard to their body temperature and heart rate during the Vierdaagse. They are tested before the march and when they return every day.

When a walker of the Vierdaagse takes part in the research, how does it affect his day?

If the walker starts at 5.00am, he will visit us first at 4.45am. Some simple tests will be completed, for example weight, temperature and heart rate. He can consume a cup of coffee and start from our research centre, instead of waiting in the queue, which may take up to thirty minutes. Alongside the route, students from the department are there to perform some measurements: heart rate, temperature, sometimes they take the collect urine; depending on the theme of the study. In the beginning, most of the walkers grumble: as they also have to check in on Sunday or Monday for some baseline tests. In the end, however, most of the walkers ask if they may participate again next year; mostly because of avoiding the queue and they tell us that they like the atmosphere in our research centre at the Wedren.

Considering this is a huge study with many people involved, how long do the preparations take and who else is involved besides yourself?

As a matter of fact the whole department helps, including many students. Choosing the theme takes place directly in the autumn. The actual preparations start around April or May. In June, many people are really busy. At an early stage we ask interns in the department to help and advertisements are put on the Intranet to find volunteers. Dependent on the theme we also work together with external partners. This year we are focusing on nutrition: does a participant of the Vierdaagse eat healthier than average? Our partners are the University of Wageningen and the hospital Gelderse Vallei in Ede. This collaboration is established partly because of my work: I work one day a week in Wageningen on the Eat2move project. Eat2move tries to answer questions like: how can we improve the recovery of top athletes with their nutrition and how can you prepare patients for surgery in order to decrease the number of complications?

> ***"Journalists of several news channels like the BBC came to Nijmegen: it was complete chaos!"***

During the Vierdaagse itself stands are set up along the route, these have to be manned at 4 am. It all requires lots of preparation and organisation but those involved are such a dedicated group and the atmosphere is great. That is what makes it possible to do such fulfilling and unique research.

Over the years, the study has also resulted in several publications. What have the reactions been to these publications so far?

Very good, Thijs Eijsvogels got his PhD on the Vierdaagse study in 2011. In the first years we concentrated on fluid balance and

Figure 1 *Maria Hopman, professor of integrative physiology at Radboudumc.*

temperature. After that we have been doing theme-based research: overweight, patients with diabetes which we have trained, heart failure patients. We have a number of publications every year.
Moreover the study has also drawn media attention, depending on the themes, and there have been some themes which attracted widespread public attention. For example, in 2007 we introduced a pill which enabled us to monitor the inner temperature. It was complete chaos that year! Journalists of several news channels (NOS, BBC, WDR) came to Nijmegen. Two years ago we studied patients over 80 years old, we got much attention that year. It fascinated people: 90-Year-Old hikers walking forty km. In 2016 the Vierdaagse will be organized for the hundredth time. That will be a fantastic year and we will definitely continue our study. I cannot tell much more about the distant future. There are always enough ideas for the study however.

What have your major findings been so far?
We have established that every year the first walking day is the most demanding on the body; participants reach the highest heart rates and get the most dehydrated. On the second, third and fourth day, the body has already adjusted to the walking, and people can handle the circumstances better.

We did not find a difference between trained and untrained walkers. In others words, there was no correlation between training and maintenance of the fluid balance. We did however find that men in general are more likely to get dehydrated, and overweight people in particular.

We would also like to ask you some questions about your work as an associate editor at Journal of Applied Physiology and Physiological Reviews. Could you tell us what that implies?
The chief editor assigns me an article and, if I consider it is good enough concerning the quality, I send it to a couple of reviewers. I receive the review reports from the reviewers, and then it is up to me to make a decision about the article. If I do not consider the article as good enough initially, then I may reject the article right away.

Could you tell us something about your passion for research?
What I like the most is the combination of creativity and curiosity to discover new things that may lead to new developments. The delightful thing about research is that you make up questions, you study in your field of interest and the results may guide towards innovative movements. The phase of interpreting your data is captivating: what is the outcome, what do the outliers tell you? Finally, the writing is also a thrilling part: you are really finishing your study.

> ***"Do not just apply statistics to your data, and interpret if your data are significant or not. Think about the outliers, they can learn you something as well."***

Could you give a tip to students who would like to develop their research skills?
Students can contact research departments during their bachelor or master studies, and enquire if they can help in a study. Then you add that experience to your curriculum vitae, especially if you are a (co) author of an article. If you are interested in doing research, you'd better look past the blinkers and use your creativity. Do not just apply statistics to your data, and interpret whether your data are significant or not. Think about the outliers, as they can also give you an insight about something as well.

Unfortunately, practicing medicine and research is increasingly about following guidelines. But you should keep on thinking creatively, dare to ask questions and not become engrossed in the statistics.

Call for Case Reports

RAMS is a journal for all (bio)medical students in Nijmegen. Not all students have the available resources to conduct large scale research. Since RAMS does not just focus on research papers, case reports can therefore be a useful way to publish in this medical journal.

The main goal of a case report is to share a valuable clinical lesson, which is perfectly in line with our aim. For a case report, students are asked to describe the symptoms, signs, diagnosis, treatment and follow up of an individual patient. Students are free to choose their own topic, therefore you do not have to discover your own Elephant Man or an entirely new syndrome. Instead, you can focus on unexpected associations between diseases or symptoms, or unexpected events in the course of observing or treating a patient. You could also produce a case report on: findings that shed new light on the possible pathogenesis of a disease or an adverse effect, the unique or rare features of a disease, unique therapeutic approaches, or a positional or quantitative variation of the anatomical structures. Therefore, the topic of a case report does not have to be a rare disease or a rare complication. Consider writing a case report about more common diagnostic or therapeutic challenges that other students might face some day during one of their internships. After all, the most important criteria are that your report is educational and entertaining.

Take into consideration gaining permission from your supervisor and an informed consent from the patient. More detailed information on how to submit a case report can be found on our website.

Submissions can be sent to submit.rams@ru.nl.

RAMS

A Word from the Chair of RAMS

A new academic year, a new curriculum and thus a new edition. Last year our medical journal, Radboud Annals of Medical Students (RAMS), has grown from an idea into reality. This year we will try to make RAMS part of the new curriculum. RAMS will offer both the new curriculum students and the old curriculum students an accessible opportunity into the world of academic science. Every (bio)medical student can do research, write an article and publish it in our journal, even if you are a first year student.
RAMS does not stop there. This year we will again offer a free summer school, master classes, symposiums and informal receptions. We are even developing a programme which we hope will eventually become a part of the curriculum. If you want to do more than just read our journal you can also join our staff as a reviewer, editor, editor-in-chief or as a board member. You can find us at ramsresearch.nl.

On behalf of the board of RAMS,

Lars Gallée, Chair

General Board

RAMS is directed by the general board, which consists of five medical students. As members of the board they frequently meet to make sure all activities run smoothly. Moreover, they are in close contact with the supervisory board and the editorial staff. If you have any questions on general, promotional or financial subjects, you can contact the general board of RAMS via vice-voorzitter.rams@ru.nl.

Editorial Board

The editorial board is responsible for the contents of the journal, from reviewing the submitted papers to their rejection or publication. Furthermore, the editorial board is in charge of writing editorials and determining the general layout. For questions concerning the content of the journal please contact the editorial staff via hoofdredactie.rams@ru.nl. To submit papers, consult the 'for authors'-section on our website or mail to submit.rams@ru.nl.

Reviewers

This is the largest group in our team. RAMS counts on the support of over twenty reviewers who have been trained by professors and teachers at the Radboudumc. With the help of specially developed masterclasses and use of their own specific knowledge, the reviewers are able to judge the submitted scientific articles.

Finished your research internship?

Publish your article in our upcoming edition! *

Send it to submit.rams@ru.nl or visit our website:

ramsresearch.nl

* Only with your supervisor's permission