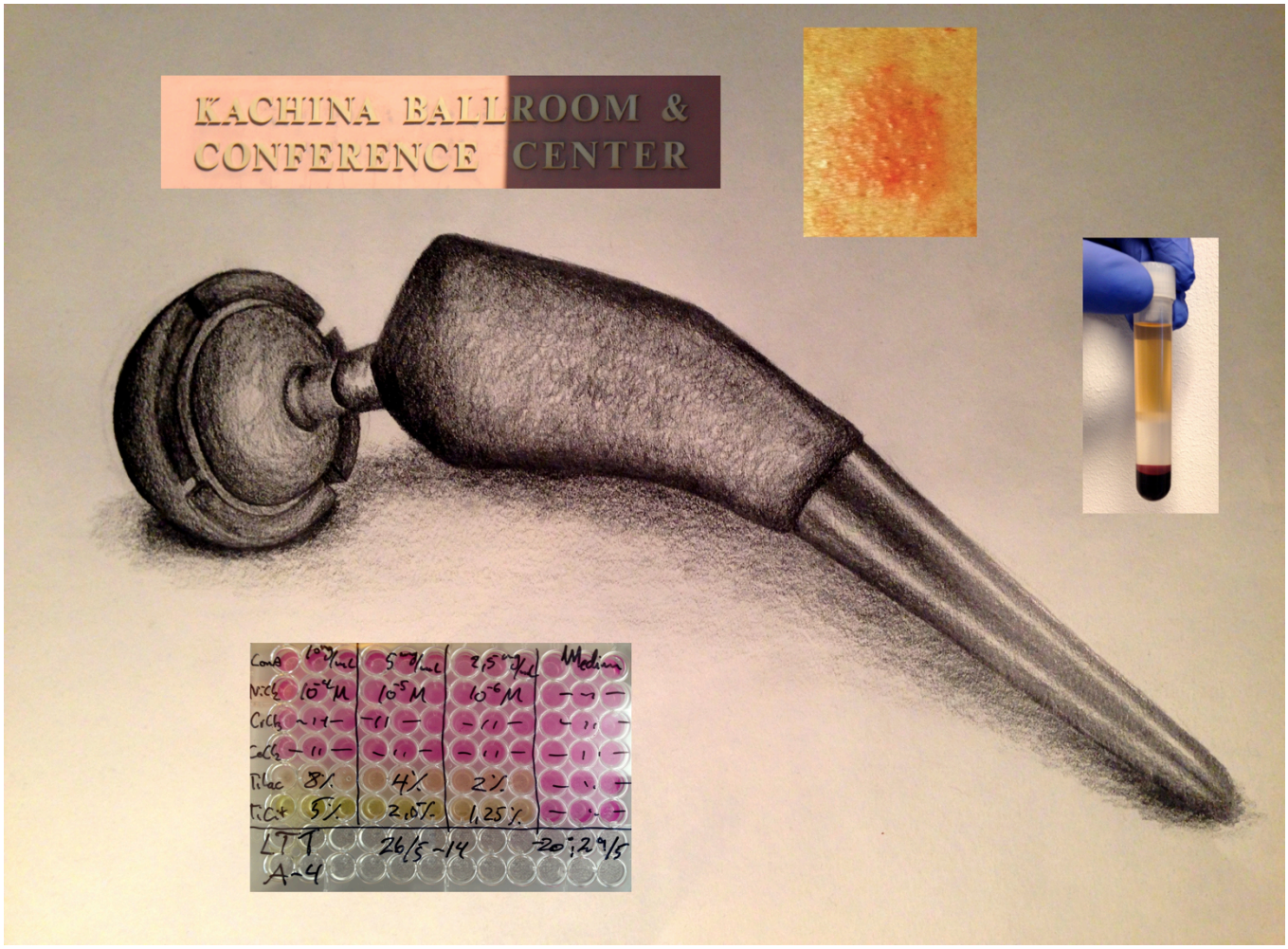
An evaluation of the immune response in hip arthroplasty patients using dermato-immunological analyses



A report of a research year

By

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Preface

My interest in implant immunology stems from a lecture on hypersensitivity in the basic course in immunology held during the 6th semester of the bachelor's degree in medicine at Aarhus University. After the lecture I approached professor Bent Deleuran and asked how come the immune system reacts to all foreign bodies introduced to the organism, but orthopaedic implants stay there and stay functioning for so long. He passionately explained that it was in fact not always the case, and went on to give me contact information on my current supervisors.

In this report I have chosen to present my data and relevant findings in a manuscript for an article. Therefore I have focused my supplementary information on a debate of the methods and the validity of the data that have come out of them, rather than debating the actual data again. Tables and figures are presented at the end of the sections that they correlate to (article manuscript and supplementary information respectively).

Main supervisors

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Abbreviations (alphabetical)

Al - Aluminium

CM – Cytokine Measurements

ConA – Concavalin A

Cpm – Counts pr. Minute

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

ICDRG – International Contact Dermatitis Research Group

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

IFN - Interferon

IL – Interleukin

IR – Irritant Reaction

LTT – Lymphocyte Transformation Test

Mo – Molybdenum

MoM – Metal on Metal (implant)

Nb – Niobium

PBMC – Peripheral Blood Monocytic Cells

PE – Polyethylene (liners)

PT – Patch Test

RPMI – Roswell Park Memorial Institute (Medium)

SI – Stimulation Index

THA – Total Hip Arthroplasty

Ti –Titanium

TNF – Tumour Necrosis Factor

V - Vanadium

Summary in English

Osteoarthritis can affect all joints in the body and the only curative treatment is total joint replacement. This procedure is both considered safe and effective dramatically increasing the patients' quality of life, or so it is in most cases. A few patients experience adverse reactions either shortly after their arthroplasty or even many years after. Though these adverse reactions can range from aseptic osteolysis to dislocations to infections, and each fraction only affects a few patients, there is still a need for understanding the mechanisms and the pathology. In this study we investigated to which degree a "normal" patient with osteoarthritis' immune system was immunological different than a patient who had experienced either dislocation or aseptic osteolysis. The aim was to evaluate the effect of metal and metal hypersensitivity in the patient groups through multiple dermato-immunological analyses.

We were not able to discriminate between the groups in a general setting. Despite low total number of patients we conclude that metal allergy doesn't seem likely to be a risk factor in the general arthroplastic patient whether be it a primary or a revision patient.

Resume på dansk

Slidgigt kan påvirke alle led i kroppen og den eneste kurative behandling er i dag operativ udskiftning af leddet. Operationen anses både for at være sikker og effektiv. Patientens livskvalitet forbedres dramatisk. Desværre opnår ganske få patienter ikke denne forbedring som følge af uønskede reaktioner der opstår enten kort tid efter operationen eller endog mange år efter. Selvom disse reaktioner rangerer vidt fra aseptisk løsning, luksationer til infektioner, og selvom kun en fraktion af patienter oplever en af disse reaktioner er der stadig et behov for at forstå mekanismerne og patologien bag. I dette studie forsøgte vi at undersøge i hvilken grad en "normal" patient med slidgigt var immunologisk anderledes end patienter med kunstige hofter som enten blev skiftet på grund af aseptisk løsning eller skiftet på grund af et mekanisk problem. Formålet var at undersøge effekten af metal og overfølsomhed overfor metaller i patientgrupperne ved brug af flere dermato-immunologiske analyser.

Med de valgte analyser var det ikke muligt at skelne mellem vores grupper. Selvom der var få patienter inkluderet, leder det os alligevel til konklusionen at metalallergi næppe er en betydende risikofaktor for den almindelige patient der skal proteseforsynes.

Manuscript for Article

Background

Total Hip Arthroplasty (THA) is an increasingly common surgical procedure normally with excellent results (Kurtz et al., 2005). Sometimes, however, even this procedure is subjugated by complications. The most common reasons for revision are aseptic loosening, bacterial infection, mechanical complications (e.g. dislocation) and trauma (Register, 2013). The etiology of aseptic loosening has yet to be fully understood (Sundfeldt et al., 2006)**.** One theory is that aseptic loosening is caused by a ("delayed") Type IV hypersensitivity reaction caused by sensitization of T-helper cells, stimulating other peripheral blood mononuclear cells (PBMCs), causing an adverse reaction at the bone-implant interphase (Thomas et al., 2009, Kwon et al., 2012, Basko-Plluska et al., 2011). Metal particles and ions are released from the implants due to wear and corrosion (Hallab et al., 2001) and have been found in hair, urine and tissue samples from around the joint. Also, a higher degree of particles seems to be apparent in patients with an unstable implant (Coleman et al., 1973). Metal ions have been shown to activate the cellular immune response (Thierse et al., 2005) and patients with an unstable Metal-on-Metal (MoM) implant are known to have a higher prevalence of metal allergy as shown on a patch test (PT) (Thomas et al., 2009). The connection between delayed type hypersensitivity and osteolysis around the implant is, however, still uncertain. A recent study has shown Th1 specific cytokines such as interferon- γ and IL-2 to be prevalent in histological samples from the bone-implant interphase (Gallo et al., 2013). These cytokines are expressed in metal allergy as well, creating a connection between metal allergy and osteolysis (Gallo et al., 2014). The question whether metal allergy precedes the osteolysis and therefore could represent a risk factor for arthroplastic surgery or whether the allergy is caused by secondary sensitization by the released metal ions. We aimed to evaluate metal allergy in patients with failing

hip implants by performing PT, lymphocyte transformation test (LTT), cytokine measurements (CM) and metal ion level status on three different hip arthroplasty related patient groups.

Materials & methods

Patch Testing

The special patch test series used in this study was provided by Smart Practice (McDowell Drive, Phoenix, Az) containing prefabricated panels with metallic compounds associated with orthopedic prostheses on Scanpor tape.

Nickel chloride (1.0%), potassium dichromate (0.054%) and cobalt chloride (0.02%) were included in the series. More notable are the different implant specific metals that were titrated down for validation of which concentration could facilitate a response. The metals with corresponding titrations were as follows: Vanadium Oxide (0.36, 0.18, 0.06, 0.02%), vanadium chloride (0.24, 0.12, 0.04, 0.013%), manganese (0.24, 0.08, 0.06, 0.0057%), Aluminum chloride (0.72, 0.38, 0.039%), molybdenum (0.12, 0.04, 0.013%), Titanium Oxylate (0.32, 0.16, 0.08, 0.04%), Titanium Dioxide (0.24%), Titanium Oxide Oxylate (2.4, 1.2, 0.6%), Titanium Lactate (0.16, 0.08, 0.04%), Titanium Citrate (0.32, 0.16, 0.08, 0.04%). Methyl Methacrylate (2%), Gentamycin Sulfate (20%) and Ferrous Chloride (2%) were tested by manually loading of a Finn chamber on Scanpor tape. Patches were applied on the upper back and were occluded for 48 hours. Readings were done at 96 hours after application (Todd et al., 1996). The patients were instructed to remove the panels after 48 hours as well as not to shower, scratch or expose to sunlight. Reactions were scored using the International Contact Dermatitis Research Group's (ICDRG) criteria (Wilkinson et al., 1970). Only definite +1, +2 and +3 reactions were regarded as positive.

Lymphocyte Transformation Test

The LTT was done on PBMCs separated from the patients' blood by the Lymphoprep (Alere A/S, 2605 Brøndby, Denmark, code 1114547) gradient technique (Yeo et al., 2009), and the PBMC cell layer was harvested and kept suspended in RPMI + 10%FBS +1%Pen/strep (Sigma-Aldrich, Denmark, codes R8758, F7524, P4333). The cells were washed thrice with medium, counted in the NucleoCounter*NC-250 (ChemoMetec, 3450 Allerød, Denmark) or, when unavailable, in a phase contrast microscope. Hereafter the cell suspension was diluted to a standard concentration of $4*10^6$Cells/mL in RPMI + 10% Autologous serum + 1%Pen/strep.

The LTT was preformed by adding 100 μ L of the metal solutions to the 96-wells plate, and afterwards adding 100 μ L of the cell dilution to each well. The metals used were $NiCl_2$ (Code: N6136, Sigma-Aldrich, Denmark), $CrCl_3$ (Code: 27096, Sigma-Aldrich, Denmark) and $CoCl_2$ (Code: 15862, Sigma-Aldrich, Denmark).

All metals were diluted and used in a 10^{-4}M, a 10^{-5}M and a 10^{-6}M solution (Kwon et al., 2010). Triplicates were done where the cell count yielded enough suspended cells to fill each well. Otherwise duplicates were done. Positive and negative controls were done using Concavalin A (ConA) (Sigma-Alrich, Denmark, code L7647) in triplicates as the positive control and nothing but growth medium (see above) as a negative control. After filling, the plate was incubated at 37°C with 5% CO_2 for 48 hours. The wells were added 1 μ Ci/well of [H_3] isotope of Thymidine, and allowed to proliferate for another 24 hours. Hereafter the plate was stored at –20°C and harvested (TomTec Harvester96, 1000 Sherman Ave, Hamden, CT.) which prepared them for counting in a scintillator (Wallac 1450 Microbeta TriLux Liquid Scintillator and Luminescence counter, PerkinElmer Life and Analytical Sciences, 710 Bridgeport Ave, Sheldon CT). Mean values for each concentration were calculated. The stimulation index (SI) was calculated by the ratio of mean counts per minute (cpm) of stimulated cells to the control (culture medium only) cultures. The SI

stimulation index was used to compare the lymphocyte proliferative (reactivity) response. Data from the concentration interval were excluded if the SI for the positive ConA controls was <2.

Cytokine Measurements

A secondary 96-wells plate was made containing the same set up as for the LTT, but only for the intermediary concentration of all the metals (10^{-5}M). This was incubated for 48 hours.

Post incubation the supernatant of approximately 100 μ L of each 3 filled wells were pooled and stored at -80°C for post stimulatory cytokine measurements. For cytokine analysis, we used a V-PLEX custom human cytokine kit (Meso Scale Discovery, 1601 Research Blvd. Rockville, MD 20850), which provided data on following human cytokines: Cytokine Panel 1 (human), IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF, IL-15, IL-17A

Metal Concentration

A plasma blood sample was sent to Vejle Hospital, Department of Clinical Biochemistry, Denmark, for trace metal concentration analysis, screening for chrome and cobalt levels before the surgery. The samples were analyzed in an ICP-MS device (Thermo Fischer Scientific Inc, 81 Wyman Street, Waltham, MA). The molecules were split creating atoms. After this, an electron was removed from the atoms and the ions were weighed in the mass spectrometer. The samples are diluted with 0.5% HNO3 with added Gallium intern standard. The detection level for ion concentration of was 10 nmol/L.

Statistics

Based on probability plots and Kolmogorov-Smirnov test normality of distribution was evaluated. Data did not follow a normal distribution data and were presented as medians with interquartile ranges and p-values less than 0.05 were considered statistically significant and compared using Mann-U-Whitney test or Kruskall-Wallis as appropriate. Statistical analyses were performed using STATA 11.0 (STATA Corp LP, College Station, Texas).

Ethics

The study was approved by the local ethics committee under journal number: 1-10-72-90-13 and by the local data supervision committee with the reference number: 2012-41-0946. No part of this study has interfered with the patients' scheduled treatment.

Results

The mean age for group A was 60.8 years, for group B 73 years, and for group C 62 years. The gender distribution was 4/2 (men/women) for the A group, 4/6 for B and 5/3 for group C. The patients' (group A and B) individual exposures from their implants are listed in Table 1. None of the patients used immunomodulation medication, had occupational metal exposure, known metal allergy towards implanted metals, or had positive Kamme-Lindberg biopsies.

Patch Testing

We did not find any statistically significant differences between group A, B and C. Positive and doubtful reactions are presented in Table 2. Of the metals used in the standard series, only one reaction to nickel (in group C) and one to chromium (in group A) was present. Vanadium showed most reactions altogether (2 positive and 17 doubtful).

Lymphocyte Transformation Test

The LTT was not able to discriminate between the three groups in a degree that was statistically significant (fig 1-3 and table 3). We found comparable levels of metal allergy with the background population.

Interestingly, the group in which the number of patients had most incidences of SI<2, was the B group. These values were, respectively, 2.99 and 2.62 to nickel, 2.49 and 2.10 to chromium and 2.33 to cobalt. The two positive reactions in group A (2.10) and C (2.35) were for cobalt and chromium, respectively.

Cytokine Profile

Post-stimulatory cytokine measurements on supernatants are presented in table 4-6. We did not find any statistically significant differences between pro-inflammatory and Th1 related cytokine levels (p-values not shown)

Metal Concentration Analysis

Metal concentration analysis was completed on 17/20 patients. The data are presented in Table 7. We found a statistically significant ($p=0.004$) difference between groups A and B with respect to chromium, as the A group had a higher degree of metal concentration than the B group. No such difference was observed with respect to cobalt ($p=0.212$).

Discussion

The concept of metal allergy leading to aseptic loosening has been debated back and forth in the literature for many years (Krecisz et al., 2006, Carlsson and Moller, 1989, Frigerio et al., 2011, Granchi et al., 2012, Rooker and Wilkinson, 1980).

In this study we investigated the incidence of metal allergy between patients with various degrees of implant failure, and patients who received an orthopedic metal implant for the first time.

We found no statistically significant association between metal allergy (PT, LTT, CM) and revision due to aseptic loosening. We found a statistically significant increased serum chromium level in patients revised due to aseptic loosening.

The PT did not yield any conclusive difference between the three groups. We observed one less definite positive reaction in the B group than the A and C group. Seen on an individual level, the most severe reactions were seen on patient A1 who both had chromium and vanadium PT reactions (figure 4-5), as well as very high chromium levels (80.2nmol/L) with exposure from a Ti-6Al-4V implant with CoCrMo femoral head.

The PT showed a diverse contact dermatitis profile across the groups. Nickel, being the most common metal sensitizer, was discovered in only 1 patient, which is low considering the patients' age group (Thyssen et al., 2009b). 1 patient in group A was positive for chromium, but more interesting is the relatively high prevalence of titanium reactions, which have also been seen by others (Muller and Valentine-Thon, 2006). 1 patient in the B group and 2 patients in the C group were positive for this. More problematic was the Vanadium chloride, which showed the highest amount of reactions. Positive and doubtful reactions amounted to 19 reactions on all groups and across all concentrations, but only two of these were positive, and they were both on the same patient. Coincidentally, this was the same patient who had chromium allergy. Doubtful and IR findings in PTs for metal are not uncommon (Fischer and Rystedt, 1985), but the surprisingly high amount that we found need further elucidation.

The LTT did not distinguish between the groups. The LTT is a quite debated *in vitro* evaluation of the adaptive immune system (Hallab et al., 2010). We were, however, able to show proliferation of the cells, as well as a few incidents of hyper proliferation, telling us that the cells stimulated with

nickel, chrome and cobalt were still active. The lack of statistically significance may be due to technical challenges. Some previous studies use a longer incubation time for their cells (Hallab et al., 2008, Kwon et al., 2010, Gustafson et al., 2014). The LTT is generally not validated and a lot of different techniques concerning metal concentration levels, incubation time and time until analysis vary from study to study (Frigerio et al., 2011, Hallab, 2004, Carlsson and Moller, 1989). All our LTT analyses were done as soon as possible after blood drawing, all within the next 4 hours, in order to ensure optimal survivability chances for the lymphocytes. It is also important to note that using the [H_3]-Thymidine incorporation method only yields a secondary measurement of proliferation, as the cell counter counts the DNA in which the thymidine has been incorporated, not the actual number of cells. Doing a pre-stimulatory analysis on the patients' serum would create data for the patients' habitual cytokine levels. These could be compared to the post-stimulatory cytokine levels presented here. This, however, would be mostly useful on an individual level, as statistical analysis on the supernatant has yielded data that did not show any statistical significance in between the groups.

Interestingly, the main group of patients with elevated ion concentrations was the A group, in which 2/3 had one or both metals elevated with levels ranging up to 80.2 nmol/L for one patient for chromium ($P = 0.004$). This may be due to aseptically loose implants experiencing micro-motion and therefore generates more wear on the implant than well-anchored implants only exhibiting dislocation. This was not reproducible in the cobalt group, as the significance test only yielded a p-value of 0.212.

We found an expected slight overrepresentation of the male gender (<60% in each group) reflecting a larger need for arthroplasties in the male gender. Furthermore, we found the mean age of revision was lower in the group with aseptic loosening contra the dislocation group indicating aseptic

loosening causing the need for revision in an earlier stage than component failure and dislocation (Register, 2013)

The lack of statistically significant associations can be explained by the small number of patients, but by choosing cases revised due to aseptic loosening as a positive control and patients receiving their first total hip arthroplasty as a negative control, we optimize our chances of proving a correlation. Furthermore, most published clinical journals in this area have relatively small sample sizes (Krecisz et al., 2006, Thomas et al., 2009) ranging up to 57 and 92 cases (Hallab et al., 2005, Kwon et al., 2010). This problem hinders significant statistical analysis and the data might be prone to random error. Due to the high number of analyses the patients in this study have undergone, however, the possibility of showing a tendency for future research is present.

In today's Denmark most patients receive uncemented implants (Lucht, 2000, Register, 2013). Previously, this was not always the case and many of our patients had a cemented implant and thus a lower exposition to metal (Kmiec et al., 2014).

It is important to realize that the arthroplastic field in orthopedics is an ever-evolving one. As such, new implants are continuously being produced and sent onto the market. These implants often vary, especially concerning alloy and bearing ranging from Co-Cr to Oxidized Zirconium (OxZr) (Dalal et al., 2012). The discontinuation of the Large Head Metal-on-Metal prostheses has certainly stemmed some of the adverse reactions contributed to wear particles (i.e. metallosis, pseudotumors etc.) (Xia et al., 2011, Kwon et al., 2010), and so has the addition of cross-linked polyethylene as a liner-component (Kremers et al., 2012). As such, we evaluated the immune system of three different patient groups undergoing THA or revision THA to see if these were immunologically different.

Conclusion

We did not find any statistically significant association between aseptic loosening and metal allergy. Despite low levels of patients, based on our results and the existing literature, it remains doubtful that there is any significant reward to gain from routinely screening for hypersensitivity in patients undergoing primary and revision hip arthroplasty. The LTT proved unable to distinguish between the patient groups in our setup, and the PT should mostly be considered in patients with a history of severe prosthesis complications and a medical history of metal allergy. The direct validity of the PT is only determinable by considerably bigger longitudinal studies. More longitudinal studies are generally needed before we can fully elucidate the relationship between metal allergy and hypersensitivity. There is especially a need need for toxicological and immunological mapping of the tissue in failing total hip arthroplasties.

Tables and figures – Relevant to the article manuscript

Table 1. – Patient prosthesis profile

Exposition	Femoral component	Composition metals	Femoral head	Acetabular component	Composition metals
A1	ZMR	Ti-6Al-4V	CoCrMo	Trilogy	Ti-6Al-4V
A2	Exeter	Orthinox (CrNiMn)	CoCrMo	Duraloc	cpTi
A3	Lubinus Monoblok	CoCrMo	CoCrMo	Lubinus Cup	Polyethylene
A4	Exeter	Orthinox (CrNiMn)	CoCrMo	Mallory Head	Ti-6Al-4V
A5	Bi-metric	Ti-6Al-4V	CoCrMo	Mallory Head	Ti-6Al-4V
A6	Bi-metric	Ti-6Al-4V	CoCrMo	ReCap	CoCrMo
B1	CLS spotorno	Ti-6Al-7Nb	CoCrMo	Trilogy	Ti-6Al-4V
B2	CLS spotorno	Ti-6Al-7Nb	CoCrMo	Trilogy	Ti-6Al-4V
B3	Biocontact	Ti-6Al-4V	Ceramic	Plasma	Ti-6Al-4V
B4	Exeter	Orthinox (CrNiMn)	CoCrMo	Pinnacle	cpTi
B5	Exeter	Orthinox (CrNiMn)	CoCrMo	Trilogy	Ti-6Al-4V
B6	Exeter	Orthinox (CrNiMn)	CoCrMo	Pinnacle	cpTi

Table 2. – Positive and doubtful patch test readings

	Group A	Group B	Group C
PT	+(+?)*	+(+?)*	+(+?)*
Metal	3(4)	2(8)	3(12)
Nickel	0(0)	0(0)	**1**(1)
Chromium	**1**(0)	0(0)	0(0)
Cobalt	0(0)	0(0)	0(0)
Titanium	0(0)	**1**(0)	**2**(0)
Vanadium	**2**(3)	0(6)	0(8)
Manganese	0(1)	**1**(2)	0(2)
Molybdenum	0(0)	0(0)	0(0)
Aluminium	0(0)	0(0)	0(1)

*Scored as positive (+) and doubtful reactions (+?)

Table 3. Hyper proliferation readings on Lymphocyte transformation test

LTT	SI>2(n)	SI>2(n)	SI>2(n)
Nickel	0(6)	2(6)	0(8)
Chromium	0(6)	2(6)	1(8)
Cobalt	1(5)	1(6)	0(8)

Table 4. Post-stimulatory cytokine measurements - Nickel

Supernatant	Group A	Group B	Group C
INF-y	581 (357-824)	597 (373-922)	537 (381-761)
IL-10	1365 (829-2698)	712 (355-1392)	561 (304-1817)
IL-12p70	386 (143-520)	381 (261-559)	160 (100-369)
IL-13	865 (676-2600)	1071 (384-1251)	512 (512 (254-1824)
IL-1beta	2093 (749-5850)	1427 (646-1816)	2294 (506-6311)
IL-2	1050 (578-1390)	1015 (478-2286)	448 (309-1313)
IL-4	384 (195-570)	359 (185-842)	220 (191,5-489)
IL-6	1982 (1134-2944)	1107 (478-10597)	486 (244-6312)
IL-8	1419139 (16041-1896953)	721887 (30006-1697956)	470272 (14433-1809932)
TNF-alpha	2173 (389-3769)	898 (354-2405)	810 (158-9229)
GM-CSF	75 (66-90)	120 (80-226)	72 (63-133)
IL-15	92 (84-97)	79 (77-80)	77 (71-88)
IL-17	51 (37-66)	75 (66-90)	63 (46-90)

Table 5. Post-stimulatory cytokine measurements Chromium

Supernatant	Group A	Group B	Group C
INF-y	924 (347-1612)	394 (320-800)	492 (396-515)
IL-10	1717 (294-3868)	784 (346-5066)	730 (320-2655)
IL-12p70	1047 (272-1359)	182 (136-310)	175 (142-243)
IL-13	3513 (978-6237)	891 (266-1405)	638 (245-1470)
IL-1beta	2084 (363-7362)	684 (411-1930)	741 (428-2267)
IL-2	1336 (483-3874)	650 (388-2742)	565 (328-3276)
IL-4	796 (258-1479)	252 (162-471)	251 (162-336)
IL-6	1989 (1102-12838)	567 (185-2097)	632 (238-1687)
IL-8	1498746 (79726-1925898)	201295 (32509-887572)	605032 (20934-1712368)
TNF-alpha	3197 (439-3883)	810 (308-1613)	969 (355-3078)
GM-CSF	78 (62-119)	75 (59-137)	72 (57-113)
IL-15	90 (89-93)	76 (64-79)	81 (77-95)
IL-17	73 (66-84)	49 (40-68)	58 (24-74)

Table 6. Post-stimulatory cytokine measurements - Cobalt

Supernatant	Group A	Group B	Group C
INF-y	556 (313-585)	570 (321-775)	686 (549-812)
IL-10	932 (285-2130)	1042 (340-1935)	1079 (613-1722)
IL-12p70	317 (273-457)	305 (84-510)	286 (164-471)
IL-13	999 (285-1790)	697 (587-1985)	493 (437-1319)
IL-1beta	768 (329-1164)	839 (329-1435)	662 (627-1575)
IL-2	1370 (305-4127)	496 (333-1731)	1211 (833-5444)
IL-4	337 (239-601)	234 (142-659)	389 (236-664)
IL-6	990 (285-9082)	473 (303-1658)	594 (436-4311)
IL-8	979350 (29421-1847013)	128026 (16852-1855477)	587885 (23530-1802204)
TNF-alpha	1399 (285-5273)	847 (337-1822)	1037 (184-1906)
GM-CSF	87 (62-224)	75 (62-114)	80 (62-171)
IL-15	93 (83-109)	83 (78-89)	87 (68-98)
IL-17	59 (54-86)	85 (67-110)	56 (46-88)

Table 7. Metal concentration analyses

Group	Chromium	Cobalt
Group A (n=6)	19 (0-60.5)	0 (0-28.3)
Group B (n=6)	0 (0-0)	0 (0-0)
Group C (n=8)	0 (0-0)	0 (0-0)

Medians with interquartile ranges.

Figure 1. LTT response - Nickel

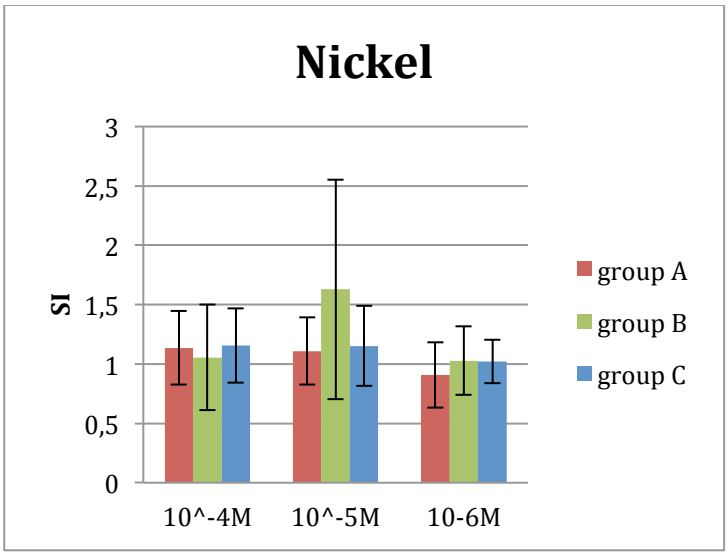


Figure 2. LTT response –Chromium

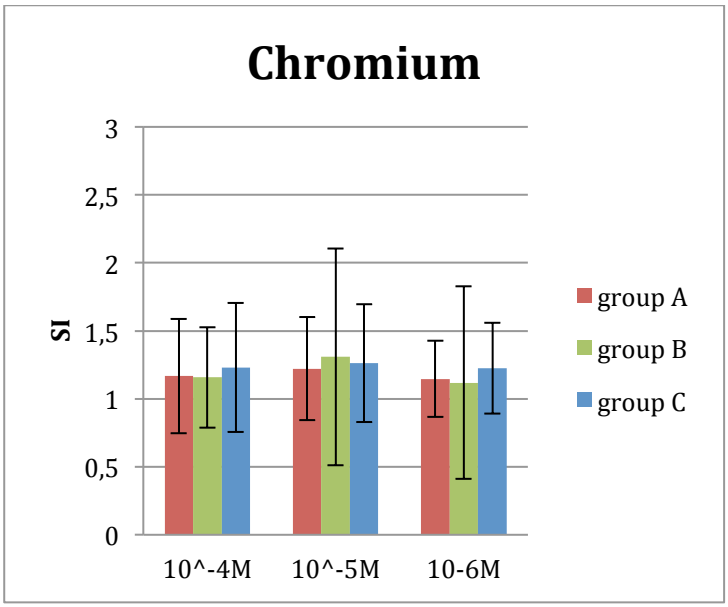


Figure 3. LTT response – Cobalt

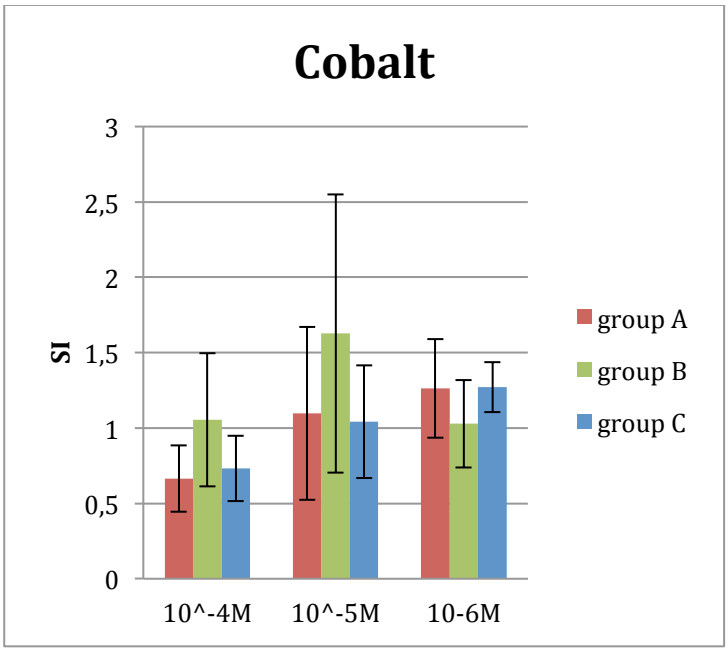


Figure 4. – Overview of patient A-1

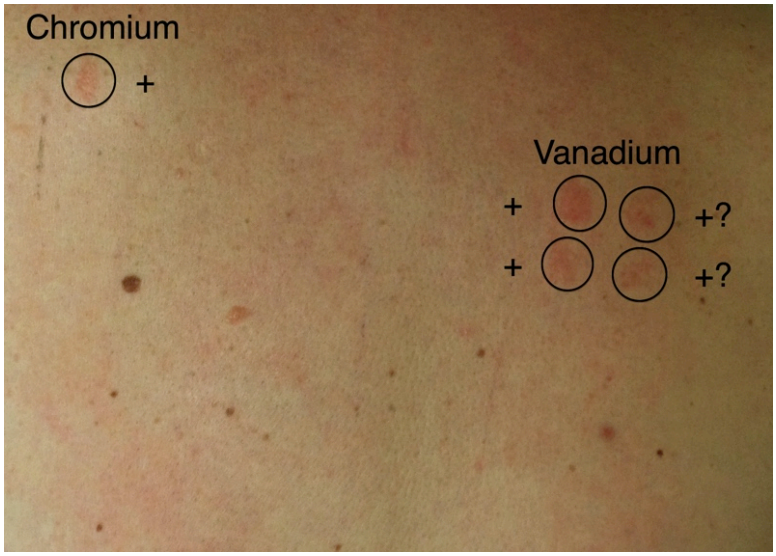
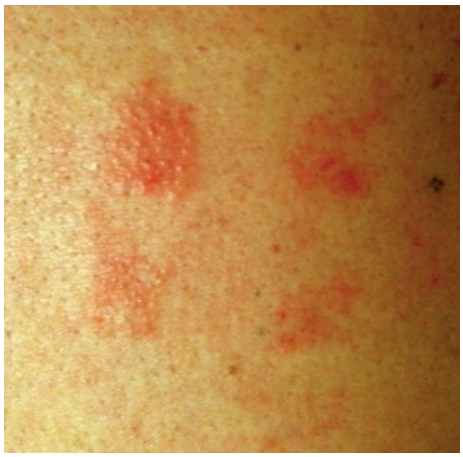


Figure 5. – Positive and doubtful reactions to Vanadium in patient A-1 (enlarged)



Supplementary Information

Courses, Conferences and Other Work

In connection with the research year the following relevant courses and conferences were attended.

Attended Courses

19th-23rd of August: Introduction to flow bench, PBMC extraction and LTT, Cluster of Immunology, Copenhagen university, Panum Institute.

16th-27th of September: Course in Patch Testing, Department of Dermatology, Aarhus University Hospital, PP. Ørumsgade, Denmark

24th-27th of October: The 3rd annual Patch Test Workshop, Phoenix Arizona

18th of November: Couse in Literature Search, Aarhus University, research year course

25th of February: Course in Introduction to Research in Health Science, Aarhus University, research year course.

2nd-4th of April: Course in Molecular Immunology and Research, Aarhus university PhD course.

Attended Conferences

14th-16th of March: Oral presentation at the Congress for Medical Pre-graduate Research, Sandbjerg Estate, Als, Denmark

9th-11th of October: Poster accepted at the European Hip Society congress 2014, Stockholm, Sweden

22nd-24th of October: Attended the Danish Orthopaedic Society 2014, Radisson Blu, Copenhagen, Denmark.

Other Scientific Work

During my research year I authored the paper:

"The association between metal allergy, total knee arthroplasty and revision.

Münch HJ, Jakobsen SS, Olesen JT, Menné T, Søballe K, Johansen JD, Thyssen JP

Acta Orthop Scand, Accepted 2014

Project overview

The role of metal allergy in the etiopathogenisis of aseptic loosening of orthopedic implants is unknown. We therefore evaluated the association between multiple immunologic tests and different implant complications to illustrate to which degree a patient with a loosened implant is immunologically different than a general hip patient. Three patient groups of eight primary operations (C), six revisions due to aseptic loosening (A) and six revisions due to dislocation/component failure (B) were included. Patients underwent patch testing, lymphocyte transformation test, cytokine measurements, and serum metal ion concentrations. We did not find any differences between PT, LTT, CM between groups A, B, or C. In group A we found one chromium and two vanadium positive PT and one cobalt positive LTT. In group B we found one titanium and one manganese positive PT reactions and two nickel and two chromium positive LTTs. In group C we found two titanium and one nickel positive PT as well as one chromium positive LTT.

Post-stimulatory cytokine measurements on pro-inflammatory cytokines did not differ significantly between the groups after either nickel, chromium or cobalt stimulation.

In group A median serum chromium was 19 nmol/l (0 – 61 nmol/l). In group B and C we found 0 nnmol/l (0 – 0) (p=0.004). We found low levels of serum cobalt and no statistically significant differences between groups. Despite low numbers of patients we conclude that there does not seem to be a significant risk of metal hypersensitivity leading directly to aseptic loosening, as tests were not able to distinguish between groups A, B and C. Metal allergy should, however, not be disregarded as a possible contributor in selected patients where a strong medical history of metal allergy is present. (i.e. high metal ion concentration level and positive PT).

Considerations on the methods and limitations of the study

Logistics

The main limitation was the low study population. Due to the unknown standard deviation on the tests analyses we preformed, we were not able to power calculate a sample size. Therefore we aimed to include 30 patients. The logistics in this study were challenging, however, and due to the high number of fixed time events the patients had to undergo, only 20 out of 30 patients were included. The most problematic logistic problem was the PT, which had to be preformed prior to the surgery. This was usually done in the patients' own home regardless of where in the country they lived. The LTT also had fixed incubation times, which further encumbered the process. Figure 1 illustrates what a patient in this study has undergone.

We had 2 patients whom we had to exclude. This was either due to an acute cancellation in the operation programme with rescheduling being impossible, or due to a case of sickness on the operation day. The resulting reduced study population is thus an unfortunate limitation that could only have been avoided by either starting earlier before training in PT and LTT was complete, or by finishing later than would have been possible due to the deadline of this report.

The Patch Test

Patch testing is the oldest and most standardized test for contact dermatitis that is readily available in the clinical setting and as such it remains the golden standard for allergy testing. For comparability to other studies in this field, it was therefore imperative that we utilized the PT as one of the analyses on the study population. There is still much debate whether a cutaneous reaction can be used as a projector for an internal response (Hallab et al., 2008, Schalock and Thyssen, 2013), but the PTs status as the most valid allergological diagnostic tool, makes it an important contributor to the data.

Choosing to do only a 96-hour reading is also a limitation of the study. The standard procedure requires readings after 72, 96 and 128 hours (Uter et al., 1996). (Todd et al., 1996) has determined, however, that metal allergens usually present with a positive reaction within 96 hours and that if only one reading should be performed, the 96-hour reading is the most important. As such, even though most reactions should be evident at the time of reading, there is undoubtedly a risk of missing otherwise positive reactions (Saino et al., 1995)

An important consideration is that many metals are considered as irritants as well as potential allergens (Fischer and Rystedt, 1985). This increases the risk of both irritant reactions (IR) and false positive reactions. We did see a high number of doubtful reactions, strengthening the previous statement

Since the patch test series was specially designed for screening of orthopaedic implants, most metals were present in different concentrations and some even in different chemical structures. This provided for a broader characteristic of the response and the possibility of evaluating a seemingly positive response as potentially a false positive response, if a reaction only was present in the low concentration and not in the higher. For the full PT series see Table 1 and for an applied PT see figure 2.

The Lymphocyte Transformation Test

The LTT is a relatively new addition to the field of implant immunology. Orthopaedic Analysis LCC 2201 W Campbell Park Dr, Suite 211, Chicago, IL have made their method readily accessible online (www.orthopaedicanalysis.com). Unfortunately the results of various experiments utilizing the LTT have produced mixed results with some authors considering the method reproducible (Valentine-Thon et al., 2006) and other remaining more sceptical (Kwon et al., 2010). Even though there are many consensual points in the different LTT methods used, there is not as such a

standardized protocol. Furthermore the analysis remains a specialized procedure, which limits its use to specialized centres. An overview of the protocol used in this study is as follows:

- Blood is drawn in 10mL EDTA-tubes and less than 4hrs later the analysis began in the laboratory
- Peripheral Blood Monocytic Cells (PBMCs) were separated from blood using the Ficoll gradient technique:
- The cells were washed in medium 3 times
- The cells were counted and approx. $4*10^6$cells/mL were seeded on a 96-wells microplate in 3 different concentrations and each concentration in triplicates.
- The plate was incubated for 48 hours before and 24 hours after adding [H_3]-Thymidine.
- The plates were stored at – 20°C before harvesting and counting cells. .
- The SI was calculated as $Mean(cpm(Metal)) / Mean(cpm(neg.control))$

Table 3 illustrates how a 96-wells LTT plate looked in the study. There could be a methodological problem in the setup as we used a much reduced incubation time before adding the [H_3]-Thymidine compared to other studies. We only used 48 hours of incubation, whereas 7 days has been the standard in other studies (Hallab, 2004). A 7-day incubation, however, could require more added growth medium due to the rapid turnover rate of lymphocytes. Furthermore we used metal concentrations based on literature (Kwon et al., 2010), but these might not have been ideal concentrations for such a short incubation time.

Importantly, the metal compounds ($NiCl_2$, $CrCl_3$ and $CoCl_2$) used, were bought specifically for in vitro stimulation of live cells. The titanium compounds (Ti Lactate and Ti Citrate) were provided by Smart Practice (Hillerød, Denmark). Ti-Lactate and citrate were chosen, as they are relatively new compounds in the PT series aimed for easier penetration of the stratum corneum. Unfortunately, the Ti compounds we used were invalidated in *in vitro* stimulation tests. Titanium is scarcely tested in

proliferation studies and it is possible that the concentration was set too high for the cells to handle (Lalor et al., 1991). Also, the chemical nature of titanium makes it very difficult to dissolve in an ionized form. We consulted with chemists at Smart Practice to optimize our solutions, but the dissolvability limited the different sub-compounds of titanium that we could use.
We preformed the LTT for 6 different solutions (pos-control, Ni, Cr, Co, Ti-lac, Ti-cit and neg-control). Theoretically, we should have done the LTT on all the same compounds that were included in the PT. There was a limit, however, to how much blood we could draw from the individual patients, and therefore how many PBMCs we could extract. Therefore we chose Ni, Cr and Co for comparability with the literature and Ti-lac and cit because they were in need of validation.

The Cytokine Measurements

Cytokine measurement analysis by ELISA or MULTIPLEX (V-plex) is a common immunologic examination. We used CM as a supplement for the LTT and aimed to find an increase in pro-inflammatory cytokines in the revision groups. Doing this analysis both on serum and the supernatant also would give an impression of the development of a cytokine response from habitual concentrations to post stimulatory concentrations. Since the supernatant cytokine levels did not show any statistical difference between the groups, however, we decided to postpone serum analysis and compare these separately at a later date. A limitation, however, was that we had a small amount of supernatant available, especially compared to the amount of serum. This creates the need to be selective in the specific cytokines to be examined were we to do a traditional ELISA analysis. Choosing a multiplex analysis over a traditional ELISA test gave us a wider array of cytokines on a small amount of material. Unfortunately the need for increased sensitivity creates the risk of false positive reactions and as such the uncertainty becomes greater. Validating the multiplex

kit is also of the utmost importance since some human patient samples might have developed autoantibodies against animal proteins in the analysis kit, which ultimately could create potential false positive responses.

Care was taken in selecting the kit. We decided on using cytokines, which were commonly expressed in inflammation and thus were comparable to the existing literature (Jensen et al., 2004, Dalal et al., 2012). We chose a kit screening for: Cytokine Panel 1 (human) IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF, IL-15, IL-17A. The kit contained a wide array of especially macrophage derived (and associated) cytokines. The kit was externally validated and seemed to be a good match for a mapping the immunologic response.

Metal Concentration Measurements

Serum metal levels are important as a reference value as it gives information about the exposure in patient groups A and B, and thus to which degree there is a basis for T-cell sensitization.

Blood samples were taken the same day as the operation before pre-operative medicine was administered. All samples were taken by bioanalysists from the department of Clinical Biochemistry at Aarhus University Hospital, Tage Hansensgade. This was done in a standardized fashion where blood is drawn for other purposes first, so that the needle is rinsed of metallic dust (Penny and Overgaard, 2010). All analyses were made at the department of Clinical Biochemistry at Vejle Hospital.

Discussion of validity and the present data

The PT gave us data that were in line with previous studies (Thyssen et al., 2009a, Granchi et al., 2012) The relevance of the patch test reactions seems unpredictable, even when we used a specialized patch test series for metal allergens on patients with osteolysis, as we had no more

positive reactions in the revision groups A and B than in the control group C. It is also established that the PT reader has to be specialized within the field. This was overcome by attending the 3rd annual patch test workshop, Phoenix, AZ, as well as visiting the local contact dermatitis clinic at the Department of Dermatology, Aarhus University Hospital, P. P Ørumsgade (Svedman et al., 2012). All patch test reactions were photo documented and at application of the test a PatchMap® (Smart Practice, Az) was drawn for easier recognition of reaction placement (Uter et al., 2007, Uter et al., 2009). After completion of the study, all PT reactions were reviewed by a dermatologist specialized in contact dermatology (JPT). All readings were done strictly according to the ICDRG criteria (Wilkinson et al., 1970) (Table 2), and emphasis was laid on determining erythema and infiltration in >95% of the allergen's panel position before regarding it as positive. This could in turn be a reason why so many doubtful reactions have been scored, some of which could have been regarded as positives in other contact dermatitis clinics where readings perhaps aren't done as strictly. We saw 14(70%) of the patients who had one or more reactions, which scored as a doubtful (+?) reaction. This is an alarmingly high number, and creates the need for addressing only a 96-hour reading as a potentially larger limitation than first believed.

If arthroplasty patients were exposed with high enough levels of metal debris, there could be a basis for a toxic inflammatory response (Scharf et al., 2014). Very possibly this inflammatory process would attract macrophages, which are known to enhance osteoclast function (Gallo et al., 2013, Hallab et al., 2008). A possible explanation for the reduced number of positive reactions in the A and B group could be that the patch test function could be altered in some of the patient groups. This is for instance seen in patients with rheumatoid arthritis and psoriasis. These patients are known to exhibit an inverse relationship between their disease status and their presentation of contact dermatitis on PT (Engkilde et al., 2012, Bangsgaard et al., 2009). Even though the pathology of these diseases are not identical with osteolysis, it is possible that the patients in the A

group are combatting inflammation, and therefore have a reduced cutaneous recognition of allergens.

A third possibility for the low level of reactions in the A and B groups is that continuous metal stimulation through wear particles could be enough to create a basis for a desensitization syndrome (Abbas and Hull, 2012). (Van Hoogstraten et al., 1991) showed that patients who had undergone oral administration of nickel at an early age had fewer reactions on PT, which could be interpreted as a lower rate of sensitisation. When looking at the B group's tendency to have fewer PT reactions, but higher LTT proliferation, this possibility could be worth exploring. It is however not unheard of to have a negative PT and still have cells that can be activated *in vitro* (Lisby et al., 1999)

To ensure the validity of the LTT data, training in correct flow bench technique was undergone at the Cluster of Immunology, Copenhagen University, Panum, as well as continuously by supervision at the laboratories where the procedure was performed. A careful log was kept during training so that post hoc supervision was also possible, and the project was not initiated until the log matched the standard protocol. Training was systematically continued until the readings were done in a standardized fashion and the PBMC suspensions could reach a concentration of $4*10^6$ cells/mL. Unfortunately the original laboratory in Aarhus did not have a 96-wells harvester or a corresponding scintillator, and therefore all test plates were frozen at -20°C. In May 2014 the lab was closed due to downsizing at the university, and all LTT work was moved to the Laboratory of Immunology and Microbiology, Aarhus University, Bartholin. All LTT work was continued according to the standard protocol, however, and only minor changes to the hardware were allowed. The data showed no significant proliferation in most patients. The cut-off for this was set to >2 SI, according to existing literature (Gustafson et al., 2014). Incubation time varies between studies, however, and so there might be some value in changing the cut-off to a lower value. Doing so would increase our findings and the validity of the protocol used, but would reduce the

comparability with other studies as well as creating a specificity problem. Another key thing to remember, is that the SI is a secondary measurement for proliferation, as it is based on how much DNA that has been incorporated in the cells, not the cells themselves. Both Titanium compounds showed fewer counts than the negative control in all patients and have as such been counted as having destroyed the cells. Most likely this is due to the difficult characteristics of titanium making it difficult to dissolve, and/or due to over-exposure from a perhaps too high concentration spectrum (Thomas et al., 2006).

The proliferative differences between our utilized metals (Ni, Cr, Co) and the titanium compounds, gave us the possibility of comparing internally with cells that had obviously experienced toxic concentrations. Using this as a reference shows and validates the protocol somewhat, as we were able to show some, if not statistically significant, proliferation in most patients.

Future perspectives

The field of implant immunology has been a research focus for many years. An important point is that the implants today are not like the implants 10 or 15 years ago, nor are the surgical procedures, the postoperative treatment or the rehabilitation. Researchers found a causal connection between debris from PE liners and aseptic loosening in the mid-90ies. This, however, is becoming a less essential challenge since the implantation of the new improved highly cross-linked polyethylene (UHMWPE) (Atkins et al., 2011). Somewhat similar is the history of MoM implants, the use of which was discontinued in the 70ties due to significant concerns as expressed by (Benson et al., 1975, Elves et al., 1975). These implants saw a renaissance due to much improved implant technology and were reintroduced as large-head MoM implants. Once again however, the MoM implants turned out problematic and in March 2012 the Danish Orthopaedic Society published an

addendum to their MoM surveillance recommendation from 2010. This addendum recommended the implants discontinued (Overgaard, 2010, Overgaard, 2012).

The relevance of future research in this field is therefore an ever-changing one as new products create new challenges, and old products sometimes resurface partially or entirely changed.

Whether or not metal allergy creates a predisposition for implant malfunction remains unclear, and the cause should be considered multifactorial since no single theory of implant malfunction so far has been able to produce any causal connection.

As such one could say that the clinical relevance in determining the pre-operative metal allergy status is currently low. However, it is important that the theory of an allergic component in implant failure is not abandoned in future research as adverse reactions are seen in certain individuals. Therefore it should not be deemed irrelevant to test a patient with severe osteolysis early after implantation for metal allergies before performing a revision arthroplasty, if no other explanation of the osteolysis can be found and the patient has a strong medical history of allergy. In case of a positive allergy evaluation, it would seem only logical that the allergens be avoided when selecting the implant to use. An important point though, is that there is a consensus among many surgeons stating that an inferior prosthesis should not be implemented, even in the case of a positive metal allergy test (Razak et al., 2013).

Conclusion

Data were not able to discriminate between the 3 patient groups in this study.

Whether or not this was due to the small sample size, methodological limitations or an actual illustration of the immune response in the groups remains speculative. There does not, however, seem to be a significant risk of type IV hypersensitivity leading to aseptic loosening in the general orthopaedic patient. The possibility of metal allergy being part of a multifactorial pathogenesis of

osteolysis, cannot be ruled out and should still be considered in patients with a very strong medical history for contact dermatitis or, most importantly, in patients with multiple arthroplastic revisions. A general fear of metal allergy foregoing arthroplasty should thus not be entertained as a predictor of a poor outcome post-implantation.

Acknowledgements

Professor Thomas Ledet and Birgitte Hviid Mumm and Professor Bent Deleuran and Marlene Hvid, Mariane Høgsbjerg Schleimann, and Bettina Bundsgaard for technically assistance.

Gitte and Vibeke at the department of Dermatology, Aarhus University Hospital, for their assistance in PT training.

Also Aarhus University for financial support and Curt Hamann, CEO of Smart Practice, Arizona, for supplying the patch test series.

Finally, Rikke, Inger, Lone and Tina and of course my supervisors both credited and uncredited, without whom none of this had been possible.

Tables and figures - relevant to the supplementary information

Table 1. The PT series

Panel 1			
Position 1	Nickel Sulfate (5.0%)	Position 7	Potassium Chromate (0.054%)
Position 2	Titanium Oxylate (0.32%)	Position 8	Molybdenum (0.12%)
Position 3	Titanium Oxylate (0.16%)	Position 9	Molybdenum (0.04%)
Position 4	Titanium Oxylate (0.08%)	Position 10	Molybdenum (0.013%)
Position 5	Titanium Oxylate (0.04%)	Position 11	Blank
Position 6	Titanium Dioxide (0.24%)	Position 12	Cobalt (0.02%)
Panel 2			
Position 1	Titanium Oxide Oxylate (2.4%)		
Position 2	Titanium Oxide Oxylate (1.2%)		
Position 3	Titanium Oxide Oxylate (0.6%)		
Panel 3			
Position 1	Aluminium Chloride (0.38%)		
Position 2	Aluminium Chloride (0.72%)		
Position 3	Aluminium Chloride (0.039%)		
Panel 4			
Position 1	Vanadium Chloride (0.24%)	Position 7	Vanadium Oxide (0.36%)
Position 2	Vanadium Chloride (0.12%)	Position 8	Vanadium Oxide (0.18%)
Position 3	Vanadium Chloride (0.04%)	Position 9	Vanadium Oxide (0.06%)
Position 4	Vanadium Chloride (0.013%)	Position 10	Vanadium Oxide (0.02%)
Position 5	Manganese (0.24%)	Position 11	Manganese (0.06%)
Position 6	Manganese (0.08%)	Position 12	Manganese (0.0057%)
Panel 5			
Position 1	Titanium Lactate (0.16%)	Position 7	Titanium Citrate (0.32%)
Position 2	Titanium Lactate (0.08%)	Position 8	Titanium Citrate (0.16%)
Position 3	Titanium Lactate (0.04%)	Position 9	Titanium Citrate (0.08%)
Position 4	Titanium Dioxide (0.24%)	Position 10	Titanium Citrate (0.04%)
Position 5	Blank	Position 11	Blank
Position 6	Blank	Position 12	Blank
Extra Panel			
Position 1	Methyl Methacrylate (2.0%)		
Position 2	Gentamycin Sulfate (20.0%)		
Position 3	Ferrous Chloride (2.0%)		

Table 2. The ICDRG standard criteria for Patch Test Scoring

Symbol	Morphology	Interpretation
-	No reaction	Negative
+?	Erythema with no infiltration	Doubtful reaction
+	Erythema, infiltration, possibly discrete papules	Weak positive reaction
++	Erythema, infiltration, papules, vesicles	Strong positive reaction
+++	Erythema, infiltration, confluent vesicles	Extreme positive reaction
IR	Different types of reactions (soap effect, vesicles, blisters, necrosis)	Irritant Reaction
Nt		Not tested

Table 3. The LTT 96wells plate configureation with concentrations.

LTT	**1**	**2**	**3**	**4***	**5***	**6***	**7**	**8**	**9**	**10/11/12**
A (ConA)	10mg/mL	10mg/mL	10mg/mL	5mg/mL*	5mg/mL*	5mg/mL*	2,5mg/mL	2,5mg/mL	2,5mg/mL	Medium
B ($NiCl_2$)	10^{-4}M	10^{-4}M	10^{-4}M	10^{-5}M*	10^{-5}M*	10^{-5}M*	10^{-6}M	10^{-6}M	10^{-6}M	Medium
C ($CrCl_3$)	10^{-4}M	10^{-4}M	10^{-4}M	10^{-5}M*	10^{-5}M*	10^{-5}M*	10^{-6}M	10^{-6}M	10^{-6}M	Medium
D ($CoCl_2$)	10^{-4}M	10^{-4}M	10^{-4}M	10^{-5}M*	10^{-5}M*	10^{-5}M*	10^{-6}M	10^{-6}M	10^{-6}M	Medium
E (Ti-Lactate)	8%	8%	8%	4.0%*	4.0%*	4.0%*	2%	2%	2%	Medium
F (Ti-Citrate)	5%	5%	5%	2,5%*	2,5%*	2,5%*	1,25%	1,25%	1,25%	Medium
G										
H										

*These concentrations were also used in a separate plate where the supernatant was extracted and pooled between the three corresponding wells. This was used in the cytokine analyses.

Table 4. Metal concentration levels, stratified by individual patients

Metal concentrations (nmol/L)	Chromium	Cobalt
A1	**80,2**	<10
A2	**15,7**	<10
A3	<10	<10
A4	<10	<10
A5	**60,5**	**28,3**
A6	**22,1**	**46,2**
B1	NT	NT
B2	NT	NT
B3	<10	<10
B4	<10	<10
B5	<10	<10
B6	<10	**15.02**
C1	NT	NT
C2	<10	<10
C3	<10	<10
C4	<10	<10
C5	<10	<10
C6	<10	<10
C7	<10	<10
C9	<10	<10

NT = Not tested

Figure 1. – Flowchart over analyses

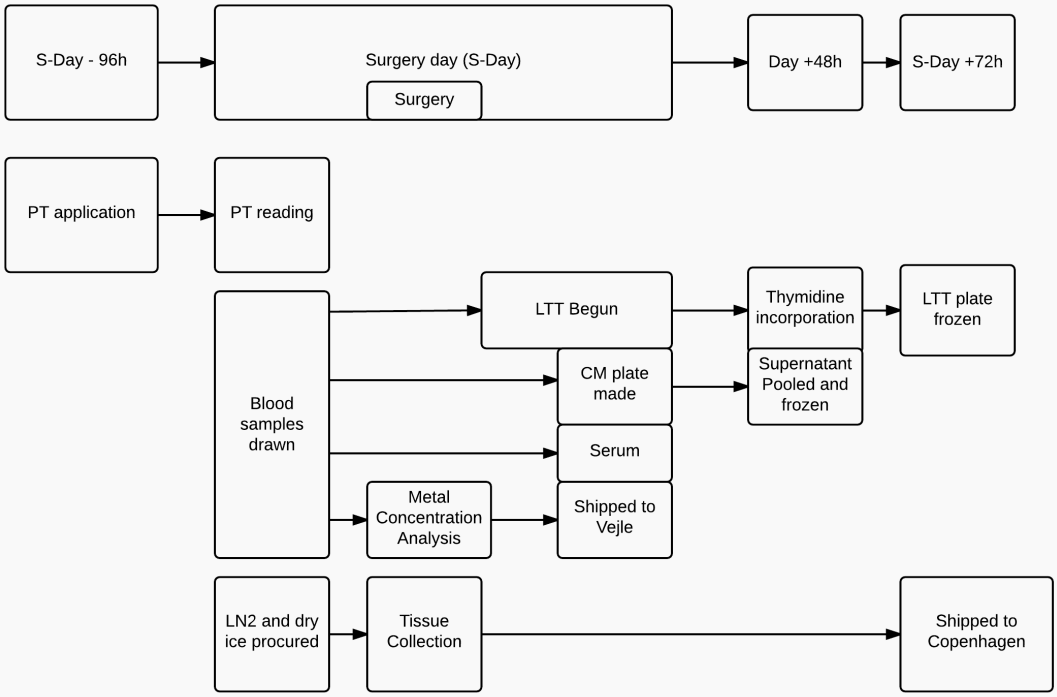
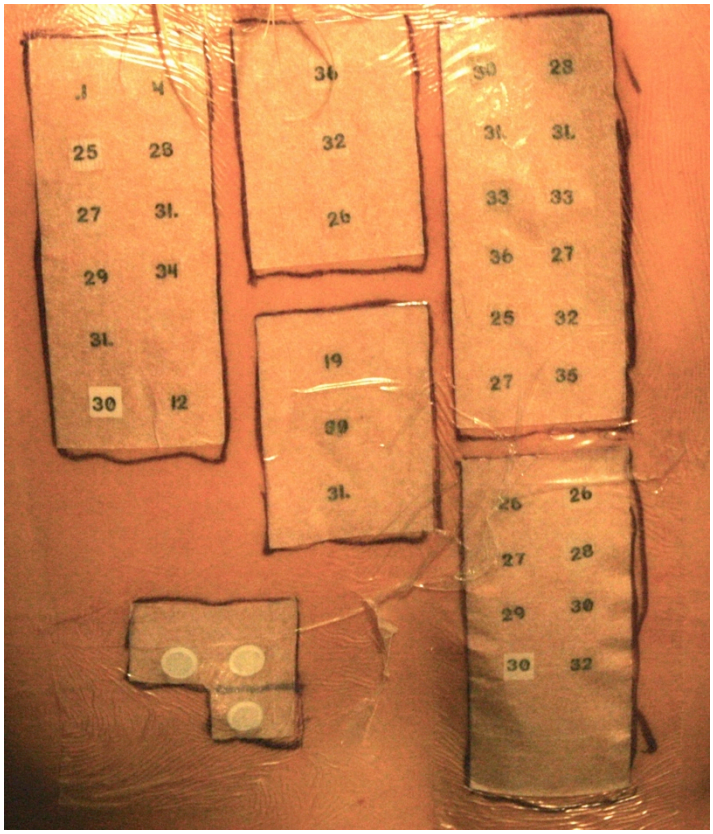


Figure 2. – Applied PT with Patch Protect®



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