4 Discussion

In January 2001 fourteen partners from five European countries started an EU-funded project to increase the efficiency and reduce the cost of bone marrow donor registries. To reach the aim of the project a three step strategy was selected. First all known alleles of the three HLA genes HLA-A, HLA-B and HLA-DRB1 were classed into groups of “frequent” and “rare” alleles, for each gene. Frequent alleles were defined as alleles which are in the most frequent HLA haplotypes in the five bone marrow donor registries involved in the MADO project. All the other alleles were defined as rare. The next step was to screen for those frequent alleles. Since the frequent alleles are very common the registries are filled with donors with frequent HLA types. It would save money if a clue of the possible HLA type could be given before high resolution typing. One of the most fatal flaws in sequencing based HLA typing is a wrong result of serological typing which leads to the use of the wrong sequencing primers. A precise pre-typing could help decrease the failure rate in other technologies and so save money and effort. One of the aims of MADO was to develop, test and establish a technology which allows screening of HLA types at high throughput for an acceptable price. All common and well established HLA analysis methods lack the possibility to be run at high throughput. The problem is less generating data than analyzing it. In last years many efforts have been made to develop and establish new technologies in the field of high throughput HLA typing. Most of them lack resolution and cost efficiency. The development of an HLA genotyping strategy is complicated due to the relatively large number of polymorphic sites in the genes analysed for HLA typing. Several HLA genotyping methodologies are available, all with some advantages and disadvantages. Direct sequencing based typing is clearly superior to other methods when high resolution is needed. However disadvantages include low throughput, time intensive scoring of alleles and ambiguities in assignment of some heterozygous allele combinations.

In this thesis a method for HLA typing is presented which combines the GOOD assay with multiplex sequence-specific primer hybridization and extension, to get

the information about microhaplotypes of a set of markers at specifically defined positions in exons 2 and 3 for the HLA class I genes HLA-A and HLA-B, and exon 2 of the HLA class II gene HLA-DRB1. Combinations of these microhaplotypes permit an estimation of the presented alleles of the sample. The GOOD assay is an approach which allows extremely high throughput SNP analysis at very low cost. It is a robust and well established procedure.

In the GOOD assay the principle of sensitivity increasing chemical modifications was implemented into a facile molecular biological procedure for generating binary, allele-specific products and sample conditioning with detection by MALDI-TOF mass spectrometry. It makes use of the multichannel detection capabilities of the MS device. Mass spectrometry reports the absolute mass value, which represents an intrinsic property of a molecule. The mass is a significantly more informative result than a fluorescently labelled DNA fragment normally used in gel-based methods. Furthermore it provides the advantage of a very fast analysis of multiple mixed compounds. The automation of this approach is fairly easy.

The GOOD assay is composed of six steps. It starts with a PCR amplification of the region of interest, which fulfils two functions. First it generates a sufficient amount of template for the allele-specific processing of the extension reaction, and second it reduces the sequence complexity of the template decreasing the risk of mispriming. There are very few SNP genotyping procedures that do not require a PCR amplification of genomic DNA. One of these PCR free approaches is the Invader assay. Another one has been introduced in an article in Nature Genetics by the group of Marc Chee where they carry out SNP genotyping without prior amplification. This assay is a genome-wide scalable microarray technology and is currently in use by Illumina, Inc.

An adaptation of these assays for HLA typing is not possible, because of the extremely high homology; up to 86 % between the different HLA class I genes and 95 % between the HLA class II genes.

The GOOD assay usually starts with a PCR in 3 µl volume, followed by addition of the next reaction mix to the PCR products. To carry out multiple GOOD assays for HLA typing would require multiple PCR of the same sample DNA and same

gene, e.g. for HLA-B eighteen PCRs would be necessary. To simplify this, the PCR was carried out in a volume of 50 µl and afterwards the product distributed into microtitre plates. This procedure has several advantages. First of all, the failure rate of the PCRs could be decreased. Usually a fairly high failure rate of the PCRs in 384-well reaction plates was observed. Failures were mostly due to liquid evaporating from the PCRs. Working in a larger volume reduces this problem. In the case of HLA-DRB1 the performance of a 50 µl PCR brings an additional advantage into the game. Co-amplification of the other HLA-DRB genes is one of the most fatal flaws for DNA-based methods of HLA-DRB1 typing and leads to false results. To avoid a mistyping of HLA-DRB1 a control marker was designed. This marker (HLADR_122r2) allows a specific identification of HLA-DRB genes. Since the reaction conditions can slightly vary from one PCR to another it could be possible that in one PCR a co-amplification of one or more of the other HLA-DRB genes is observed and for another reaction of the same sample not. This was avoided by performing a PCR in a larger volume and distribution into aliquots. In this way it was assured that for each marker the same PCR amplicon of a sample was present. The PCR is followed by a dNTP degradation step with shrimp alkaline phosphatase. This step is necessary for successful performance of the primer extension reaction. The extension reaction is performed with a primer that anneals immediately next to the SNP. The primer is then extended by a $\alpha$-S-ddNTP complimentary to the SNP. By replacing Thermosequenase with the DNA polymerase TMA 31 FS the SAP digest theoretically could be removed from the protocol, which would reduce the cost of the method. The TMA 31 FS enzyme has a significantly higher specificity for ddNTPs. However it was shown that the signal quality of the extension products was much better with SAP digestion then without. This lead to the decision to keep the SAP digestion step. Thus the TMA 31 FS DNA polymerase lost its advantage for cost reduction, which was assumed by removing of the SAP digestion step, and a low-priced Thermosequenase (ThermiPol DNA polymerase) was used in its place. The extension reaction is followed by a reduction step of the extension primer to a core sequence of the last four or five bases at the 3’- end. The reduction is carried out with a 5’ – 3’ phosphodiesterase. Finally the negatively charged backbone of the oligonucleotide is neutralized by an alkylation step and the final product analyzed with MALDI-TOF-MS. The
neutralization by alkylation is possible because the last three to four bases at the 3’-end of the extension primers are interconnected by phosphorothioate bridges. A further modification of the extension primer is a charge tag carried by the last but one base from the 3’-end. This charge tag carries a fixed positive charge, which allows sensitive analysis of the product by MALDI-TOF-MS. As was shown by Gut and Beck, charge tagging and charge neutralisation of the DNA backbone results in a dramatic increase in sensitivity together with a decrease in susceptibility to form adducts with constituents\textsuperscript{120,121}.

An additional advantage of the charge tag is the option of mass shifts, which allows the unambiguous assignment of the allele products in multiplex primer extension reactions, if the naturally occurring sequence diversity is not sufficient to establish unique masses for all alleles. For this thesis a set of five charge tag molecules were used (Table 7 in Section 2.8.1). These charge tags allow a shifting of the masses of an extension primer over a range of 56 Da in steps of 14 Da. However, a decrease of the efficiency of the primer extension reaction correlating with the size of the charge tag molecule was observed. The heaviest charge tag (CT +28) showed the lowest efficiency of all charge tags, and CT (0) the highest followed by CT (-14) and CT (-28). This probably results from the differences in the structures these molecules assume in the active centre of the DNA polymerase. Close interactions between template, primer and active site residues of a subunit of the enzyme were demonstrated by crystallisation for several types of DNA polymerase\textsuperscript{122}. Further the possible degree of automation compensates for the effort required for the multi-step procedure of the GOOD assay.

Based on the GOOD assay the first idea was to use SNP genotyping by mass spectrometry to identify frequent HLA alleles of the loci HLA-A, HLA-B and HLA-DRB1. Therefore a specific set of polymorphisms in exon 2 and 3 of the class I genes, and in exon 2 of the class II were chosen. To find these sets of polymorphisms an algorithm was created. The algorithm and the corresponding software are described in detail in section 2.14.3.

The first experiments showed a few difficulties in the use of GOOD assay to perform genotyping in the HLA region. The extremely high polymorphism content in this region makes conventional primer design almost impossible. The

\textsuperscript{120} Gut, I.G. and Beck, S., 1995, Nucleic Acid Research, Vol. 23: 1367 - 1373
\textsuperscript{121} Gut, I.G. et al., 1997, Rapid Communications in Mass spectrometry, Vol. 11: 43 - 50
fact that the Thermosequenase, which is used for the primer extension, does not tolerate mismatches within the last four bases from the 3’-end of the extension primer helps to solve this problem. This discrimination of the polymerase made it possible to use pools of primers for the same polymorphism. For each known sequence, which could possibly appear at the position of interest, a primer was designed. All primers for one marker were pooled and added into the same extension reaction. With this short DNA sequence elements of four to five bases of selected loci in the gene sequence can be generated. These constitute microhaplotypes.

To select the most informative markers, which are needed to be genotype for identification of HLA alleles, the single base polymorphisms in the genes of the HLA region were translated into microhaplotypes by a numerical code. These coded microhaplotypes were used to select a set of markers. The selection criteria based on microhaplotypes are slightly different then from the single base polymorphisms based marker selection. With the microhaplotype based selection almost all markers are equally important. They work as a set. The SNP based selection starts with the most informative marker. The most informative markers for HLA-A is HLAA_98_r2 and for HLA-B it is HLAB_539_f1. The informativity of the other markers is lower. Identical to microhaplotype based markers they work as a set with the first markers contributing more weight.

With the presented method an estimation of the HLA type can be made even with an imperfect data set. In section 3.5.1 “HLA type estimation with missing data points” was shown that as few as four out of ten markers can provide a very clear estimation of the HLA-DRB1 type. Clearly this depends on which marker is missing and what the degree of informativity of the missing marker for the allele carried by the individual is. However, the method is not primarily designed for high resolution HLA typing. It was designed as a screening procedure with a potential of reasonably high resolution. In some cases resolution of 99.99 % likelihood of a four-digit HLA type for both parental alleles could be reached. The method presented in this thesis could be used for fast and precise high throughput screening for HLA types in donor registries.

For 95 % of 655 individuals which were analysed in the context of the MADO project by this method a HLA-DRB1 type could be estimated. This estimation was made with the data set of maximally eight markers out of ten that were
attempted. To make an estimation based on these results, the frequencies of the alleles, published by Middelton et al. [www.allelefrequencies.net](http://www.allelefrequencies.net), were used. (see also section 2.14.5) Since these frequencies are calculated for populations with different origins the likelihood of the types could be specified, based on the regional and ethnic origins of the donor DNA. For example the frequency of the allele DRB1*1501 for the population England Caucasoid is 28.2% while in Greece it is only 8%. This has big impact on the likelihood of presence of this allele. In the MADO project only samples from the western European population were used.

The number of known HLA alleles is constantly growing, because every year new alleles are discovered. Therefore it will be necessary to update the set of markers in the future. However, due the separation between frequent and rare alleles the set of selected markers should suffice for quite some time, because the members of the group of frequent alleles will not change. All new discovered alleles are rare alleles, and so the resolution of this presented method will decrease with time.

4.1 Comparison of microhaplotyping by the GOOD assay and by other SNP genotyping methods

In the context of this thesis the question should be asked whether there are alternative SNP genotyping approaches which can be adapted for HLA typing applying microhaplotyping.

There are a few SNP genotyping approaches with a high degree of automation and throughput. There is for example the MassARRAY\(^\text{123}\) assay and the PinPoint\(^\text{124}\) Assay. The MassARRAY assay is quite similar to the GOOD assay. This assay also uses primer extension reaction and product identification by MALDI-TOF-MS. One drawback of the MassARRAY assay is the need of a purification step, which increased the complexity of automation. However, Jurinke et al.\(^\text{125}\) were able to show that the MassARRAY assay is a very powerful method of high throughput SNP genotyping. This assay uses an extension primer that anneals right next to the SNP. By multibase extension with a combination of

\(^{123}\text{www.sequenom.com}\)


complementary dNTPs and ddNTPs specific products are created. These products were analyzed by MALDI-TOF-MS using HPA (3-hydroxy picolinic acid) matrix. This matrix is very good for DNA fragments up to a length of 30 bases with the disadvantage of comparably low resolution. This makes it difficult to separate molecules with a small mass difference, e.g. mass differences between A and T (9 Da). This is the reason MassARRAY assay uses for the extension a combination of dNTPs and chain-terminating ddNTPs. The separation of alleles of a SNP is 289 – 329 Da. This way they are easy to analyze. The MassARRAY assay can not be used for microhaplotyping because of the length of the extension primer. On one hand it promises higher informativity, since the sequence length is up to 25 bases instead of four or five, on the other hand it increase the risk of misinterpretations. For example, a mismatch close to the 5’-end of the primer needs very stringent hybridization conditions to avoid false hybridization results. With up to ten and more extension primers per reaction and multiple SNPs in the sequences of these primers this task is extremely difficult to fulfil. At the 3’-end the specificity is controlled by the DNA polymerase. If the problem of hybridization condition can be solved it might be possible to use the MassARRAY assay also for microhaplotyping. However the ratio of effort and effect is highly outbalanced against the effect. A further problem with the MassARRAY assay is that it is developed for bi-allelic SNPs. This is a general problem for all SNP genotyping platforms. Most often a SNP is a bi-allelic system. This is different in the MHC where also four–allelic polymorphisms exist. The GOOD Assay is an exception in SNP typing technologies. It is highly flexible in terms of multi-allelic polymorphisms and can accommodate SNPs that lie close together.

4.2 Comparison of HLA typing by SBT and microhaplotyping

655 samples were analyzed with the here presented microhaplotyping method. These samples were selected by the donor registries involved in the project. Of these samples 198 individuals were also typed by SBT. SBT is considered the “Golden Standard” for HLA typing procedures. This method provides the highest resolution of all HLA typing technologies, with the added advantage that it can identify new alleles. There are only a few methods, which are able to detect
unknown alleles (e.g. RSCA) but they do not have the power to identify new alleles. Every new method has to be compared with the “Golden Standard”. One of the goals of the MADO project was to establish new methods for the screening for HLA types. To prove the ability to estimate the correct HLA types a ringtest was carried out. The SBT procedures were done in HLA typing laboratories in Vienna and Montpellier. As the results in section 3.5 and 5.5 show, for all 198 cases the correct HLA types were estimated by the microhaplotyping method. This demonstrates that HLA type screening by microhaplotyping is a very powerful technique. This is also shown by the fact that even with incomplete sets of data a very precise estimation of the possible HLA type of the individuals can be provided. Depending on the marker, in some cases even one marker out of ten suffices for a precise estimation of the HLA type. For the samples out of the 655 analyzed for this project and thesis, where no SBT results were available, it can be assumed, that the microhaplotyping results are largely correct.

The choice of method thus depends mainly on the number of samples and the availability of instrumentation. Due to its possible degree of automation the microhaplotyping method has its advantages when it comes to the analysis of a large number of samples. When information about the exact HLA type are needed, SBT should be the method of choice. However, the costs for the SBT are significantly higher compared to the single nucleotide primer extension reaction in combination with MALDI mass spectrometry, which is used for the microhaplotyping method.

Other difficulties are ambiguous typing combinations. These combinations in SBT give identical sequencing traces or in cases of microhaplotyping identical microhaplotypes combinations. For instance, an individual with the HLA-DRB1 type DRB1*0408 and DRB1*1107 can not be distinguished from an individual with the HLA-DRB1 type DRB1*0422 and DRB1*1126 (see example in table 50).

<table>
<thead>
<tr>
<th>Alleles</th>
<th>DRB1_122</th>
<th>DRB1_125</th>
<th>DRB1_196</th>
<th>DRB1_197</th>
<th>DRB1_227</th>
<th>DRB1_261</th>
<th>DRB1_286</th>
<th>DRB1_299</th>
<th>DRB1_308</th>
<th>DRB1_341</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*0408</td>
<td>AACAT</td>
<td>ATGA</td>
<td>GAGT</td>
<td>ACCT</td>
<td>AGTA</td>
<td>CGAG</td>
<td>GACC</td>
<td>AGAG</td>
<td>CGGT</td>
<td>GGCT</td>
</tr>
<tr>
<td>DRB1*1107</td>
<td>CGTCT</td>
<td>CTGA</td>
<td>GAGT</td>
<td>ACCT</td>
<td>AGTT</td>
<td>GGAG</td>
<td>GACC</td>
<td>AGAA</td>
<td>GGCT</td>
<td>GGCT</td>
</tr>
<tr>
<td>DRB1*1126</td>
<td>CGTCT</td>
<td>CTGA</td>
<td>GAGT</td>
<td>ACCT</td>
<td>AGTT</td>
<td>GGAG</td>
<td>GACC</td>
<td>AGAG</td>
<td>CGGT</td>
<td>GGGT</td>
</tr>
<tr>
<td>DRB1*0422</td>
<td>AACAT</td>
<td>ATGA</td>
<td>GAGT</td>
<td>ACCT</td>
<td>AGTA</td>
<td>CGAG</td>
<td>GACC</td>
<td>AGAA</td>
<td>GGCT</td>
<td>GGGT</td>
</tr>
</tbody>
</table>

*Table 48: Example of an ambiguous typing*
The consensus sequences of both individuals are identical. An allele-specific PCR amplification could help to solve this situation. At the first polymorphic position at either the 5’-end or the 3’-end an allele-specific PCR primer could be used for this PCR. The resulting PCR products can then be retyped. The retypes give the phase of the microhaplotypes for one of the alleles. This effort would makes sense if one of the type combinations provides the perfect match for a patient. As for the screening the prediction that the given HLA-DRB1 type is one of those combinations is sufficient, since the result of four alleles out of over 700, in the case of HLA-B, is a very good result.

In some cases even the same microhaplotypes are in identical phases, and only sequencing of the allele-specific PCR can separate the alleles. Few alleles have the identical sequences over the exon 2 and 3 for HLA class I genes, and exon 3 for HLA class II genes. This is for instance the case for HLA-A, A*0211 and A*0269, for HLA-B, B*4801 and B*4809 and for HLA-DRB1, DRB1*120101, DRB1*1206 and DRB1*1210. In these cases there is no chance to separate them without information about the other exons.

In samples microhaplotyped for this thesis no sample with ambiguous typings could be found. This could be due the fact, that mostly rare alleles are involved in ambiguous types. The use of allele-frequencies can also help to overcome this task. For instance for the example in table 50, the HLA-DRB1 type DRB1*0408/DRB1*1107 is the most likely of the two types, since the frequency of DRB1*0408 is 0.38 %. The frequencies of the three others is below 0.01 % in the here used populations. But only an allele-specific typing can tell which one is the actual one.

4.3 Software

One of the greatest technical problems for this thesis was data handling. The MHC is still a “moving target”, which means every year new alleles are discovered, with new polymorphic positions. Therefore a flexible strategy of data analysis was necessary. In the framework of this thesis a package of software was developed in close cooperation with the informatics department of the CNG. This package helps the user to select suitable markers for the identification of the alleles of the different HLA genes, analyze of genotypes and to estimate the HLA types. In this thesis the focus was only on the transplantation relevant HLA genes.
(HLA-DRB1, HLA-A and HLA-B). Since there are many other HLA genes of importance for some disease susceptibilities (e.g. HLA-C and psoriasis) it was important to develop also some software tools, which helped to adapt the method of microhaplotyping for other questions, as long the input files follow the matrix formats, which are explained in chapter 1.14 “Software developed and used for HLA typing”.

Not only selecting the right markers is of importance, since the microhaplotyping is to be used in high throughput, also the analysis of the generated data sets is essential. Nowadays the methods in molecular biology are generally very effective in data generating, e.g. Illumina is a genotyping platform able to generate more than 2 million genotypes per day. Without very powerful software these data would not be of use. For HLA typing by microhaplotyping and mass spectrometry similar problems are encountered. For example, if a set of 1000 samples is screened for their HLA types at HLA-A, -B and –DRB1, a total number of 44 000 microhaplotype reactions have to be carried out. Expecting the full set of data points, the same number of spectra has to be read. With an average reading time of 1 hour for 384 spectra, one technician would need more than 14 working days to read all the spectra for these 1000 samples. However, the analysis of generated data sets is the most time consuming part of all HLA typing methods. The software “Helixir” in combination with “HLA-Families” are very powerful tools. With these complete analysis can be done in less than a day.

“Helixir” is a tool for peak identification in mass spectra. Usually it seems to be quite easy to create a tool which is able to read spectra and identify the peaks automatically. This was not quite the case. The main difficulty was the tolerance of the mass spectrometer. In some cases the measured masses where shifted by 2-3 Da due to calibration. In these cases the software needed to be able to identify the correct peak and call the correct microhaplotype. These problems are generated by differences of the MALDI targets, differences in matrix crystallization and a number of other factors that are difficult to control. These problems will be solved in the future by the further development of the hardware, e.g. MALDI-TOF-MS has become more precise, matrices are becoming more specific for the analytes. The spectra quality will increase which makes automated peak identification much easier.
It can be said that dealing with a huge amount of data is a problem for all HLA typing methods. In all cases it is the most time and effort intensive part of a method. For example, for sequence based typing it is necessary to read the traces from the sequencers and the sequences have to be compared with known sequences in a database. Recently David Sayer at the Royal Perth Hospital\textsuperscript{126,127} created a tool, which allows the automatic sequence identification and comparison. This decreases the time needed for HLA typing by SBT enormously and make it a more effective method in terms of throughput.

Even all these new software tools can not replace the intelligence of the human brain. In some cases the computer makes the “wrong” decisions, which would lead to a false estimation of the HLA type, and that would be fatal. Therefore quality control can not be replaced.

4.4 Perspectives

As the GOOD assay for microhaplotyping is highly flexible new genes, such as HLA-C, HLA-G and HLA-DQ, could easily be analysed on the platform. Many human diseases with a genetic component are strongly associated with HLA antigens. A comprehensive list of HLA alleles and disease association is given by Shiina et al (2004)\textsuperscript{128}. For example, strong association between HLA-B27 and ankylosing spondylitis (AS) was demonstrated. 96 % of the patients with AS carry the HLA-B27 allele. Another example of a strong association between human diseases and HLA antigens is Psoriasis vulgaris. This disease has a strong association with HLA-Cw*06. The presented method for HLA typing could be used for screening for groups of disease associated HLA alleles. For instance, the markers HLAB_103f1, HLAB_206f1 and HLAB_167r2 together allow the identification of all alleles of HLA-B*27. The combination of microhaplotypes ACCT for the marker HLAB_103f1, GAGA for the marker HLAB_206f1 and TGTTC for marker HLAB_167r2, are unique for the alleles of the HLA-B*27 group.

The limit of resolution in HLA allele identification could be solved by increasing the number of markers and/or combining microhaplotyping by the GOOD assay

\textsuperscript{126} Sayer, D. et al.; 2004, Tissue Antigens, Vol. 64 : 556 - 565

\textsuperscript{127} David Sayer 2

with allele-specific PCR amplification. The use of allele-specific amplification in combination with the GOOD assay for molecular haplotyping was recently described by Tost et al\textsuperscript{129}. One envisioned strategy, which could be adopted to carry out the procedure, is, all samples are first microhaplotyped for each marker and molecular haplotyping of the microhaplotypes is only performed on samples if two or more marker sites on the PCR fragment are heterozygous. Using the allele-specific PCR products as the templates for the microhaplotyping procedure, both parental HLA alleles can be unambiguously identified.

For this further developments would have to be implemented, particularly concerning sample control and laboratory information management systems. It would be a method for high resolution HLA typing, but with a very low throughput because the potential of automation is low.

4.5 A few comments to the cost of HLA typing by microhaplo-typing

Surprising as it may seem to discuss the economical power of a procedure in an academic thesis like this, but it was defined as an objective to develop a low-cost method for screening for HLA types. Therefore some comments to the cost of the

\textsuperscript{129} Tost, J. et al.; 2002, Nucleic Acid Research, Vol. 30: e96
method should be made. The organisation of bone-marrow-donor registries includes the screening of thousands of samples every year at a huge cost. The GOOD assay provides an attractive solution for SNP genotyping, and therefore for the here presented microhaplotyping method. First of all it is a purification free assay. For other SNP genotyping methods such as MassARRAY the purification contributes a substantial part of the total cost. The major part of the costs for the GOOD assay are the enzymes. About 60% of the cost derives from the two DNA polymerases required for the GOOD assay. The contribution of robots and mass spectrometers to cost is very difficult to estimate, since it depends on the level of throughput. The higher the throughput the lower the contribution. The same is true for staff cost. The staff cost only increases the price for the assay if new markers have to be developed. However, all calculations rest sketchy. A calculation of the definite final price per donor is not yet possible. A pessimistic estimation is that HLA-A and HLA-B could cost 10 Euros each, while HLA-DRB1 would be about 5 Euros all included. In comparison SBT costs in excess of 50 Euros for each of the three genes. The pre-screen with this microhaplotyping approach would allow selecting potential donors for registries with at least four times greater coverage, which over time might result in four times more HLA types being represented in registries. However, as mentioned this is with a pessimistic price calculation. With an optimistic price calculation coverage might increase to ten times higher.