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N-glycans in liver-secreted and immunoglobulin-derived protein fractions

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Abstract

N-glycosylation of proteins provides a rich source of information on liver disease progression because majority of serum glycoproteins, with the exception of immunoglobulins, are secreted by the liver. In this report, we present results of an optimized workflow for MALDI-TOF analysis of permethylated N-glycans detached from serum proteins and separated into liver secreted and immunoglobulin fractions. We have compared relative intensities of N-glycans in 23 healthy controls and 23 cirrhosis patients. We were able to detect 82 N-glycans associated primarily with liver secreted glycoproteins, 54 N-glycans in the protein G bound fraction and 52 N-glycans in the fraction bound to protein A. The N-glycan composition of the fractions differed substantially, independent of liver disease. The relative abundance of approximately 53% N-glycans in all fractions was significantly altered in the cirrhotic liver. The removal of immunoglobulins allowed detection of an increase in a series of high mannose and hybrid N-glycans associated with the liver secreted protein fraction.

Keywords

N-glycosylation; Mass spectrometry; Serum; Liver disease

1. Introduction

Chronic liver disease is on the rise in the United States and worldwide with viral hepatitis B or C infections, alcohol consumption, and nonalcoholic steatohepatitis (NASH) representing the main causes [1]. Progressive scarring of the liver leads eventually to cirrhosis, the major cause of death in chronic liver disease; people with liver cirrhosis have also an increased risk of developing hepatocellular carcinoma (HCC) [2]. Pathophysiology of cirrhosis is not fully understood but it is known that glycosylation of proteins changes in liver disease [3]. Glycosylation is a complex posttranslational modification [4,5] with a profound functional impact on biological processes [6,7]. Changes in N-glycosylation of proteins associated with premalignant liver diseases received an increased attention in recent years [8-10]. These

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studies strongly suggest that detailed characterization of N-glycans has the potential to provide improved tools for the management of liver diseases.

With a few exceptions, such as albumin and C-reactive protein, liver secreted proteins are N-glycosylated. The liver secreted N-glycoproteins are expected to reflect the changes in liver cirrhosis; however, recent literature points to changes in the glycosylation of immunoglobulins (Ig) [8-10], the most abundant class of glycoproteins in serum that originates in the cells of the immune system, as indicators of liver disease [11]. The function of Ig heavily depends on their glycosylation status [12]. It has been shown that the composition and glycosylation of IgA, IgM, and IgG change in chronic liver disease [13-16]. The results of Klein et al. show that the major differences associated with the development of cirrhosis are attributed to the N-glycosylation of Ig [16]. Mehta et al. used reactivity of fucosylated agalacto IgG to the AAL lectin as a test for fibrosis and cirrhosis [10]. Vanderschaeghe et al. showed that the bisecting fucosylated glycans of Fibro- and Cirrho-tests are associated with Ig [8]. However, the analysis of isolated liver secreted glycoproteins indicates that their glycosylation status changes in cirrhosis as well [17,18].

We have used MALDI-TOF analysis of permethylated N-glycans [19,20] for the study of liver diseases in our previous studies [21,22]. This method allows relative quantification of tens to hundreds of N-glycans in serum but does not distinguish N-glycans associated with Ig or the liver secreted glycoproteins. In this paper, we describe an optimized workflow which allowed us to compare N-glycans of 23 healthy individuals and 23 patients with liver cirrhosis, in proteins fractionated into two fractions of Ig and a fraction of liver secreted proteins. The results show that the depletion of Ig allows detection of changes in a series of hybrid and high mannose N-glycans associated with the enriched liver secreted protein fraction.

2. Experimental section

2.1. Materials

2,5-dihydroxybenzoic acid (39319), sodium hydroxide (01209BH), trifluoroacetic acid (T6508), acetonitrile (34998), chloroform (C-2432), iodomethane (06416ME), sodium chloride (D-5545) and water (270733) were purchased from Sigma-Aldrich (St. Louis, MO). Proteus protein G (PUR015, lot 221009) and A (PUR007, lot 281009) microspin columns were from AbD Serotech, Kidlington, UK, DMSO (327182500) was from Acros Organics (Pittsburgh, PA), tC18 Sep-Pak 50 mg cartridge (WAT054960) were obtained from Waters (Milford, MA). Charcoal solid-phase extraction column (744300) was from Harvard Apparatus (Hamden, CT). Protein N-Glycosidase F (PNGase F, P0705L, 0360907-7) was from New England BioLabs (Ipswich, MA).

2.2. Study population and sample collection

A total of 23 patients with liver cirrhosis and 23 healthy volunteers were analyzed. All participants were enrolled under protocols approved by the Georgetown University Institutional Review Board. Patients were enrolled as part of a study at Georgetown University Hospital, Department of Hepatology and Liver Transplantation, Washington DC. Basic demographic information such as age, race and gender was acquired through an administered questionnaire. Clinical data for the cirrhotic patients were extracted from medical charts. All subjects donated a blood sample and 20 of the 23 healthy controls provided 4 blood samples within a year, in 2–4 month intervals, to allow analysis of the variability of the N-glycans in the same person over time. The remaining 3 disease free subjects provided three samples. Serum samples were aliquoted and stored at –80 °C till analysis. All analyses were carried out at second thaw.

2.3. Fractionation of serum proteins

Serum was fractionated on Proteus protein G and A microspin columns according to manufacturer's suggestions with the following minor adjustments. Serum (10–30 μ L) was diluted with binding buffer A (0.1 M Na_2HPO_4 , pH 7.4, 1.5 M NaCl) as summarized in Scheme 1. The sample was loaded on the protein G spin column in two cycles, 6 minutes at 240 rcf each, washed with 3×90 μ L buffer A (2 minutes, 1800 rcf), and the flow through (FT) and washes were combined for loading onto a protein A spin column. The protein A spin column was processed as above. The FT and washes from the protein A column were combined as the FT fraction (740 μ L total volume). The protein G spin column was further loaded with 30 μ L of serum in 170 μ L Buffer A. The protein G FT and wash were loaded on the protein A column as above. The A and G spin columns were further washed three times with 650 μ L buffer A and the bound proteins were eluted with elution buffer B (4×150 μ L, 0.2 M Glycine pH 2.5) directly into a tube with 65 μ L of neutralization buffer C to adjust the final pH to 7.5. The three elution cycles were combined and we refer to proteins bound to the protein G and A columns as 'G' and 'A' fractions, while the unbound FT fraction (from 10 μ L of serum) is referred to as 'FT'. In addition to the fractionated serum samples, we have also carried out analysis of a 10 μ L aliquot of unfractionated serum as described below.

2.4. Analysis of N-glycans

The handling of the three fractions, or unfractionated serum, was performed according to a published protocol with slight modifications [20]. Briefly, sample volumes were adjusted with 25 mM ammonium bicarbonate, reduced with dithiothreitol and alkylated with iodoacetamide. N-glycans were detached with PNGase F, 600 U, overnight at 37 °C. The deglycosylated proteins were precipitated at 90 °C for 10 minutes and further removed on a C18 cartridge (tC18, 100 mg, Waters, Milford, MA). The N-glycans were further cleaned on an activated charcoal solid-phase extraction column (Harvard Apparatus, Hamden, CT, USA). The N-glycans trapped on the activated charcoal were eluted in four volumes of a 250- μ L of 50% acetonitrile/water (v/v) containing 0.1% TFA. The combined eluents were dried under vacuum and the released N-glycans were permethylated on sodium hydroxide beads (reagent grade, Sigma-Aldrich, St. Louis, MO) in a solution consisting of 79.3% DMSO, 19.5% iodomethane, and 1.2% water. The optimum amounts of water and iodomethane was determined experimentally. The reaction was carried out in a capped vial at room temperature for 20 minutes and reagents were collected by a brief 10 second centrifugation at 5 000 rpm that removes the hydroxide beads. The permethylated samples were immediately extracted by a liquid-liquid extraction using 400- μ L aliquots of chloroform and water. The chloroform layer was further back-extracted with three 1-ml volumes of water and dried under vacuum.

2.5. MALDI-TOF mass spectrometry

The dried permethylated samples were resuspended in a 50:50 methanol:water solution. Each sample (0.5 μ L) was spotted directly on a MALDI plate and mixed with an equal volume of DHB matrix (10 mg/ml DHB in 50:50 methanol:sodium acetate, 2 mM). The sample spots were dried under vacuum to achieve uniform crystallization. Spectra were acquired on an Applied Biosystems 4800 Mass Analyzer (AB Sciex, Framingham, MA) equipped with a Nd:YAG 355-nm laser. MALDI spectra were recorded in the positive ion mode as permethylation eliminates the negative charge normally associated with sialylated glycans.

2.6. Data processing and analysis

Raw spectra were exported as text files and processed using an *in house* software modifying our previously published spectral processing methods [22,23]. The MALDI-TOF spectra

were calibrated on masses of a set of previously identified N-glycans [20,21,24] and exported as text files for further processing. We eliminated a binning step which was found to distort intensity-ratios of the N-glycan isotope clusters. Instead, the spectra were smoothed by the Savitsky–Golay algorithm [25], de-noised by Daubechies D20 wavelet transform [25,26], and the baseline was corrected by removing low frequency nodes using FFT convolution/deconvolution. Similar to recent publications, the presence of previously identified N-glycans was determined by matching the theoretical distribution of their isotopic clusters to the observed spectra and resolving peak integral overlaps using iterative prediction, correction procedure [24,27]. The detected N-glycans were subtracted from the spectrum and the remaining isotopic clusters, with intensity above a predefined cutoff and present in more than 20% of the analyzed spectra, were interpreted as unknown N-glycans. The identified peaks were normalized by scaling the total peak intensities to 100.

All analyses were performed using SAS software, version 9 (SAS Institute Inc., Cary, NC). We used *t*-test and Wilcoxon rank sum test to determine differences in glycan abundance between healthy individuals and patients with cirrhosis. All *p* values were two-sided, adjusted by Holm's multiple comparison procedure as needed. Pearson correlation was used to estimate correlations between glycan intensities and globulin. We have used Mixed procedure for repeated measures, adjusted by Bonferroni method, to compare intensities of 9 glycans with highest fold difference between cirrhosis and control in the FT fraction; the healthy controls were sampled and analyzed at four different time points. The association of peak intensities with age, gender, and race was analyzed by the Generalized estimating equations (GEE) method.

3. Results

The goal of our study was to evaluate changes in the N-glycosylation of liver secreted proteins. To this end, we have analyzed serum samples of 23 disease free controls and 23 patients with liver cirrhosis (Table 1). Majority of the cirrhotic patients were of viral HCV etiology and had a mean Model of end stage liver disease (MELD) score of 13. Our results show that N-glycans detached from the unfractionated serum proteins are dominated by decrease in the biantennary sialylated glycan *m/z* 2792.4 and increase in the biantennary agalacto core fucosylated glycan *m/z* 1835.9, as reported previously [9] (Fig. 1). We have also observed an increase in the bisecting fucosylated glycans, the type of N-glycans selected previously as part of the GlycoFibro and GlycoCirrho tests [8]. It was pointed out that the above N-glycans are associated primarily with immunoglobulins [16].

More than half of the cirrhotic patients in our study have elevated globulins (Table 1). When we stratify the cirrhotic patients into the groups of normal (2.3–3.5 g/dl), elevated (3.6–4.8 g/dl), and high (>4.8 g/dl) globulin, we observe a strong association of the glycans with the titers (Fig. 2). Overall, 28 of the 85 N-glycan peaks in the unfractionated serum correlate with globulin. We have therefore used a combined protein G and A depletion strategy to enrich the liver secreted glycoproteins (Scheme 1). This expansion of the previously described MALDI-TOF analysis of permethylated N-glycans [20] allows examination of the glycosylation changes associated with liver secreted proteins otherwise dominated by the N-glycans associated with Ig [16]. Our results show that the glycans detected in the combined Ig fractions substantially differ from the glycans in the FT fraction and glycans in the unfractionated serum (Fig. 3).

The Ig associated glycans represent a significant portion of the N-glycans in the unfractionated serum with some N-glycans detected only in the Ig fraction, as reported recently [9,28].

Specifically, the biantennary core fucosylated agalacto (m/z 1835.9) and monogalactosylated (m/z 2040.0) glycans were virtually undetectable in the spectra of the FT fraction of serum which supports the efficiency of the protein G and A double depletion strategy. The glycans with m/z 1835.9 and 2040.0 were also differentially abundant in the G fraction of healthy and cirrhotic subjects with m/z 1835.9 increasing relative to 2040.0 as the core fucosylated glycans shift with cirrhosis toward agalacto forms.

Because the literature points out that IgM and IgA classes of globulins, besides IgG, are important in the development of liver cirrhosis, we further fractionated the globulins into two layers. This is achieved by successive passage of the serum proteins through the protein G and protein A affinity resins which generates the FT fraction (primarily liver secreted) and two Ig fractions bound to the G and A proteins (Scheme 1). Protein G binds IgG1-4; protein A binds all the IgG subclasses, except IgG3, and IgA, IgD, IgE and IgM. The sequential trapping of Ig on protein G and protein A resins minimizes the carryover of Ig into the FT fraction and allows a separate analysis of the Ig fractions. Since the order of loading in our analysis is protein G followed by the A spin column, the protein G binds predominantly IgGs and protein A binds the remaining Ig classes. Based on the known concentrations of globulins in serum, we expect that the majority of Ig bound in the A fraction are IgMs and IgAs. Because IgMs have also multiple glycosylation sites, they are likely to represent the major contribution to the A layer especially in the case of disease with an HCV etiology [29]. The throughput of approximately 96 samples in one week is sufficient for such studies of medium throughput as the one reported here. In addition, the two individual fractions of Ig can be examined at the same time.

In the fractionated samples, we have detected overall 82 N-glycans in the FT fraction, 54 in the G fraction, and 52 in the A fraction (Supplemental Table 1). The Ig layers contain primarily glycans m/z <3000, mostly biantennary complex structures and high mannose glycans. The G fraction in cirrhotics is dominated by the appearance of agalacto core fucosylated glycans. The A layer is in our hands characterized by the monosialylated biantennary glycan m/z 2431.2 and high mannose glycans (Supplemental Table 1). A comparison of the N-glycans of the 3 glycoprotein layers (FT, G and A) in healthy versus cirrhotic participants showed significant differences in the abundance of glycans across the three fractions. We have observed a major shift in the distribution of relative intensities ranging from 68% of N-glycans in the FT layers to 33% of the N-glycans in the G layer (Table 2). The focus of this presentation is on the description of the procedure allowing the analysis of the immunoglobulin depleted glycoproteins which are primarily secreted by the liver. The flow through fraction is therefore discussed further.

With the immunoglobulins removed, we begin to observe glycosylation changes in the liver secreted fraction. The most interesting observation is an increase in high mannose and hybrid glycans in the FT (liver secreted) protein fraction (Fig. 4). Decreases in other glycans summarized in the supplemental tables may be equally important; however, we find the increase in an entire series of high mannose and hybrid glycans most interesting and focus on this novel finding. We have compared N-glycans in the cirrhosis group ($n=23$) to the healthy controls ($n=23$) sampled at four different time points. This demonstrates that the observed increase in the cirrhotic group is not due to random fluctuation in the N-glycosylation of liver secreted glycoproteins. The repeat measurements in the disease free groups are consistent with median intra person coefficient of variation (across the four repeats) of 17% and inter person coefficient of variation of 46% (among 23 participants). The intensities of the nine N-glycans are not significantly different between the draws in healthy controls except for two of the 45 comparisons (Fig. 4). In contrast, all nine N-glycans are significantly elevated in the cirrhotic group. Because the cirrhotics are a slightly older group with more Caucasian males, we have used the GEE models to investigate the

effects of age, gender and race on the nine N-glycans from the FT fraction. The analysis shows only two significant associations; glycan m/z 2029.0 is associated with gender ($p=0.009$) and glycan m/z 2390.2 is associated with age ($p=0.048$). The intensity of 2029.0 increases 1.3 times ($p<0.0001$) in connection with cirrhosis when adjusted for gender and the influence of gender becomes insignificant ($p=0.54$). The m/z 2390.0 increases 1.5 times ($p<0.0001$) with cirrhosis when adjusted for age and the intensity increases only 1.004 times with age when adjusted for cirrhosis ($p=0.05$). The analysis shows that for these nine glycans the age, race, and gender are of marginal significance compared to the disease group. This demonstrates that the series of hybrid and high mannose N-glycans are elevated in liver secreted glycoproteins in connection with cirrhosis.

4. Discussion

Chronic liver disease is a growing worldwide problem [1]. Progressive changes in liver diseases of various etiologies lead to cirrhosis, the major cause of death and a risk factor for the development of HCC [2]. Complications of liver biopsy support the need for non-invasive diagnosis of the liver diseases. The quantification of N-glycosylation of Ig [10] and detached N-glycans [8] represent an important step in this direction. The goal of our study was to evaluate changes in the N-glycosylation of liver secreted proteins using an optimized workflow for the analysis of detached N-glycans.

Considerable evidence indicates that changes in the N-glycosylation of proteins occur in liver diseases [30]. Early studies of the protein N-glycosylation showed changes in sialylation [31,32]. Increased fucosylation of haptoglobin was reported in alcoholic liver disease [33,34] and α 1-acid glycoprotein and serum cholinesterase fucosylation status were reported to diagnose liver cirrhosis [35,36]. Using mass spectrometric techniques, Morelle et al. identified three groups of N-glycan modifications in alcoholic liver cirrhosis [28]. The major changes included the presence of bisecting GlcNAc, the increase in α -1,6 fucosylated structures and the presence of neutral agalactosylated oligosaccharides [28]. A quantitative analysis of select N-glycans showed convincingly that the agalacto fucosylated N-glycans, associated primarily with Ig, can be used to detect cirrhosis [8]. We decided to examine further the N-glycans associated with liver secreted N-glycoproteins, because studies of isolated proteins show that the glycosylation cascade is modified in the development of liver cirrhosis [17,18].

To achieve this goal, we adjusted a workflow for the analysis of permethylated N-glycans [20] used previously in our laboratory to study glycosylation in HCC [21]. Analysis of detached N-glycans has become an established tool for the characterization of protein glycosylation [37,38]. Chemical or enzymatic methods for glycan detachment were described but N-glycans are typically harvested from isolated proteins [19,20] or complex protein mixtures [21] with PNGaseF. The selection of methods for subsequent glycan analysis depends on the starting material, sensitivity of detection, need for characterization of structural details, and quantitative aims. Fluorescent labeling of glycans in combination with HILIC chromatography [39] or capillary electrophoresis [40] allows an efficient fractionation and quantification. Structural characterization of the glycans is achieved by exoglycosidase digestion [41] and mass spectrometric analysis [42]. Mass spectrometry, in general, has become very useful for both structural analysis and quantification [11,43]. Mass spectrometry allows analysis of small quantities of unmodified N-glycans [44] but derivatization is often used to improve the stability, ionization efficiency, and fragmentation of the oligosaccharides [20,45]. A combination of methods is typically needed to achieve complete characterization of a sample. Extensive efforts from a number of groups have established *in house* libraries of glycans in specific tissue and disease context [46-49].

In this study, we have separated liver secreted N-glycoproteins from Ig by a two-step Protein G and A enrichment (Scheme 1). The N-glycans known to be associated with Ig are efficiently removed (Fig. 3). We have shown that some glycan structures (m/z 1835.9, 2040.0, 2244.1, 2285.1, 2489.2) are detected predominantly in the Ig fractions. The G fraction was significantly enriched with glycans of m/z 1835.9 and 2040.0, the N-glycans recently described as IgG specific in connection with the alcoholic cirrhosis of the liver [16,50]. Our population of cirrhotics consists primarily of HCV etiology. With the limited sample size, it was not possible that we carry out a conclusive analysis by etiology but the increase in the N-glycan m/z 1835.9 dominates the unfractionated cirrhotic spectra as well. While the above is the most striking change, the protein A bound and the FT (liver secreted) fractions change substantially as well. This is most clearly seen after removal of the IgG on the protein G resin.

We do not have information on the exact class of Ig associated with the differences in the A fraction. Both IgM and IgA are glycosylated, present in this fraction, and modified in the progression of liver disease. Klein et al. reported minor changes in N-glycans detached from the serum fraction depleted of immunoglobulins; major differences in their analysis were attributed to the changes in the glycosylation of IgA [9,16]. We do not know at this point whether changes in our population are associated with IgA or IgM but we have detected the most relevant differences in the liver secreted N-glycoprotein fraction. The difference, compared to the study of Klein et al., could be possibly related to the dominant HCV etiology in our study.

Two classes of N-glycans, hybrid and high mannose, are significantly increased in liver secreted glycoproteins in cirrhosis (Fig. 4). It is possible that the reorganization of the liver structure and the influence of HCV infection on the host glycosylation apparatus lead to the release of a higher portion of N-glycoproteins associated with these incompletely developed forms. The N-glycans of liver secreted N-glycoproteins are mainly of the complex type but it is known that some abundant proteins, like alpha-2-macroglobulin or complement 3 and 4, carry high mannose glycans [51,52]. It is also plausible that the hybrid glycans representing a partially complete N-glycosylation are higher in the cirrhotic liver cells. The fact that we observe an increase of an entire series of the high mannose and hybrid glycans further points to a consistent effect on these pathways. The approximately 2-fold increase is quite high and outside the range of variability of our measurements and, more importantly, outside the variability of repeat sampling in the disease free controls (Fig. 4).

The influence of demographic factors such as age, gender, and race is not well understood. The only study we are aware of described the variability and heritability of N-glycan structures in the plasma proteins [53]. The analysis of the sources of variability in N-glycosylation in this population identified small (up to 10%) but significant effect of age and gender on the N-glycan distribution [53]. We have therefore examined whether the increases in the high mannose and hybrid N-glycans could be affected by age, race, or gender differences. We have observed only minor influence of the demographic factors and conclude that the high mannose and hybrid glycans are upregulated in connection with the development of cirrhosis. This expands the changes in N-glycosylation of proteins reported to develop in the context of liver damage and will allow further exploration of the mechanisms that lead to the progression of the disease.

5. Conclusion

In conclusion, we have established a method for the analysis of enzymatically detached permethylated N-glycans in serum fractionated into liver secreted and Ig derived fractions. We have shown fraction-specific serum alterations in N-glycosylation. A consistent

upregulation of a series of high mannose and hybrid N-glycans was observed in the fraction of liver secreted glycoproteins. This is in line with the fact that majority of serum proteins are liver secreted N-glycoproteins that reflect the pathophysiology of the organ. We demonstrate that the separation of Ig and liver secreted fractions provides a rich source of information that has the potential to improve our understanding of liver disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Major N-glycans observed in a typical MALDI-TOF spectrum of the cirrhosis and control groups.

Fig. 2.
Association of N-glycans with immunoglobulins.

Fig. 4.

Boxplots of nine N-glycans with highest differences between cirrhosis and controls in the FT fraction; serum samples of the controls were collected at four different time points.

**Significantly different between cirrhosis and controls; *Significantly different among one of the control groups.

Scheme 1.

Separation of serum into a liver secreted protein fraction (FT) and two Ig fractions bound to protein A (A) and protein G (G), respectively.

Table 1

Basic characteristics of the study population.

	Healthy (n=23)	Cirrhotic (n=23)
Age, mean (SD)	48 (9)	57 (8)
Male (%)	65%	78%
Race (%)		
Caucasian	35%	52%
African American	39%	17%
Other	26%	31%
Etiology (%)		
Viral (HCV, HBV)	N/A	57%
Alcoholic		17%
Other		26%
Globulin (gm/dl)		
Normal (2.3–3.5)	N/A	35%
High (3.6–4.8)		55%
Very high (>4.8)		10%
MELD score, mean (SD)	N/A	12.7 (5.1)

Table 2

Number of N-glycans up- and down-regulated in cirrhosis in each of the FT, G, and A fractions; *p*-values adjusted by Holm's multiple comparison procedure.

Change	FT (<i>n</i> =82 glycans) <i>N</i> , %		G (<i>n</i> =54 glycans) <i>N</i> , %		A (<i>n</i> =52 glycans) <i>N</i> , %	
	Up	Down	Up	Down	Up	Down
<2 fold	6 (7%)	9 (11%)	2 (4%)	2 (4%)	1 (2%)	3 (6%)
2- to 5-fold	6 (7%)	25 (30%)	1 (2%)	12 (22%)	1 (2%)	15 (28%)
>5 fold	0	10 (12%)	0	1 (2%)	0	12 (23%)
Total	58 (68%)		18 (33%)		32 (61%)	