Acceptable changes in quality attributes of glycosylated biopharmaceuticals

To the Editor:

Since the first marketing approvals of recombinant biopharmaceuticals, the question of which changes in quality attributes, which comprise identity, strength and purity, are acceptable in the life cycle of these products without changing the product label has been debated extensively[1]. This question is especially important in the context of manufacturing process changes, which happen quite frequently and for various reasons (e.g., process improvements, scale changes or site transfers). Although companies and health authorities have been managing these quality changes for many years based on the principle that changes in quality attributes can be accepted only if they do not alter safety and efficacy, the lack of peer-reviewed data in the public domain has limited debates about product quality and variation to a discussion of principles rather than specifics. Here, we present a study that looks at variation in three major marketed biologics, the purpose of which is to provide more transparency and to anchor the debate about acceptable changes in quality attributes on a firmer factual footing. Identifying such variations in quality attributes could help not only biotech companies in their development efforts but also the medical and scientific communities in understanding these products. By analyzing the quality profiles of the glycosylated recombinant therapeutic proteins Aranesp (darbepoetin alfa), Rituxan/Mabthera (rituximab) and Enbrel (etanercept) sourced from the market between 2007 and 2010, our data thus provide examples of acceptable variations for products that have remained on the market with unchanged product labels.

Glycosylated proteins are complex molecules and even a well-controlled product may consist of several hundred or more glycoforms having the same amino acid sequence but different glycan composition. When making these products, the manufacturer has to deliver a consistent product quality to guarantee a reproducible clinical performance. Current analytical methods allow the detection of even small changes in quality attributes and can therefore enable sensitive monitoring of the batch-to-batch consistency and variability of the manufacturing process. Several different factors may account for changes in quality attributes. The first is the inherent batch-to-batch variability in the manufacturing process. Second, process drifts can lead to gradual changes of attributes. Such drifting events are not desired and normally trigger further investigations and corrective actions, or even redevelopment activities to ensure process consistency. Finally, larger and abrupt changes in quality attributes can occur after implementation of manufacturing process changes, which are all too common in the pharmaceutical industry. Although manufacturers try to prevent associated changes in quality attributes, such changes cannot be avoided in every case.

Changes in the biologics manufacturing process are tightly regulated by the health authorities. Manufacturers need to demonstrate that the process change does not alter the clinical safety or efficacy of the biologic product. The evaluation of such changes follows a comparability exercise between the pre- and post-change product, which is focused on the quality level and sometimes, depending on the magnitude of the change and the existing product understanding, also requires comparative data on the preclinical and clinical levels. The principles of the comparability exercise are regulated in guidelines, such as the International Conference on Harmonisation (ICH) Q5E (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf), which acknowledges that "the demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety or efficacy of the drug product."

Comparability decisions are difficult to make and require the complete assessment of the existing process and product including the knowledge of structure-function relationships. Others[1] have already acknowledged that a collection of data would be extremely valuable to come to a more informed design and improved assessment of comparability studies. And yet, very little data on commercialized products can be found in the public domain. In an attempt to at least partially fill this gap, we have analyzed multiple batches of Aranesp, Rituxan/Mabthera and Enbrel to study the variability in the quality attributes of modern therapeutic proteins currently on the market. The data were generated as described in the **Supplementary Methods**, using the materials listed in **Supplementary Tables 1** and **2**.

The active pharmaceutical ingredient of Aranesp, darbepoetin alfa, is an erythropoiesis-stimulating protein. It represents an engineered analog of human erythropoietin. It differs from endogenous erythropoietin mainly by an alteration of the amino acid sequence that introduces two additional *N*-glycosylation sites, which results in an elongated half-life *in vivo*. The biological activity and clinical effect of erythropoietins is influenced by the glycosylation profile, which needs to be tightly controlled during production[2].

We have characterized commercial batches sourced in the European Union (EU) by capillary zone electrophoresis, which separates isoforms with different charges resulting from varying numbers of sialic acids per molecule (**Fig. 1** and **Supplementary Fig. 1**). The *in vivo* biological activity is known to be dependent on the number of sialic acid units per molecule, which is a result of the available sialylation sites, the antennarity of the *N*-glycans and the completeness of sialylation[3].

The expiry dates of the tested batches span a range from November 2008 to April 2011. We found a change of the isoform

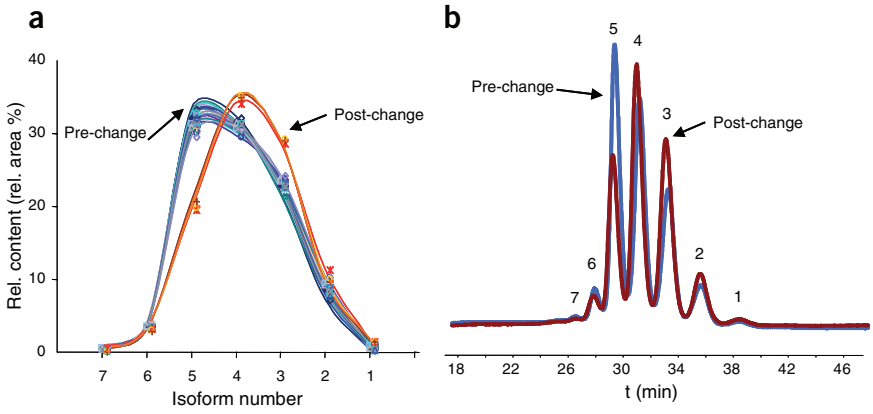

Figure 1 Comparison of the pre- and post-change Aranesp batches measured by capillary zone electrophoresis. (**a**) Relative content of the individual isoforms of the pre-change (n = 18) and the post-change (n = 4) batches. (**b**) Representative electropherograms; peaks are labeled with the isoform number.

distribution between two sets of batches (**Fig. 1a**,**b**). The batches expiring up to April 2010 showed a higher sialylation rate than the batches expiring after September 2010. The average amount of the more highly sialylated isoform number 5 decreased by an average of 10%, whereas the less sialylated isoforms increased 3% (isoform number 4) and 5% (isoform number 3). However, each of the two groups showed a very high batch-to-batch consistency (**Fig. 1a**). The magnitude might indicate that this change is a consequence of a manufacturing process change. Indeed, in 2008 the European Medicines Agency approved a major process change of Aranesp based on an extensive comparability exercise[4]. However, although the time frames are matching, we have no conclusive evidence that our data reflect this published process change.

Rituxan/Mabthera contains a chimeric IgG1 monoclonal antibody against the B-cell surface antigen CD20 (rituximab) as the active ingredient. It is mainly used in the treatment of B-cell malignancies and rheumatoid arthritis. The glycosylation-dependent Fc effector functions are essential contributors to its therapeutic mode of action and clinical efficacy[5,6].

We have characterized commercial batches of Rituxan/Mabthera with expiry dates from September 2007 to October 2011 using glycan mapping, cation exchange chromatography (CEX) and antibody-dependent cellular cytotoxicity (ADCC) *in vitro* bioactivity (**Fig. 2**). In 2008, an abrupt change in the quality profile became apparent for batches with expiry dates in 2010 or later. The most obvious difference was found in the amount of the C-terminal lysine and N-terminal glutamine variants when analyzed by cation exchange chromatography (**Fig. 2a**,**b** and **Supplementary Fig. 2a**,**c**). These variants elute as basic variants after the main peak. The post-change batches contain a much smaller amount of these basic variants whose relative amounts are reduced from ~30–50% to ~10%. It should be noted that lysine and glutamine heterogeneity is common for monoclonal

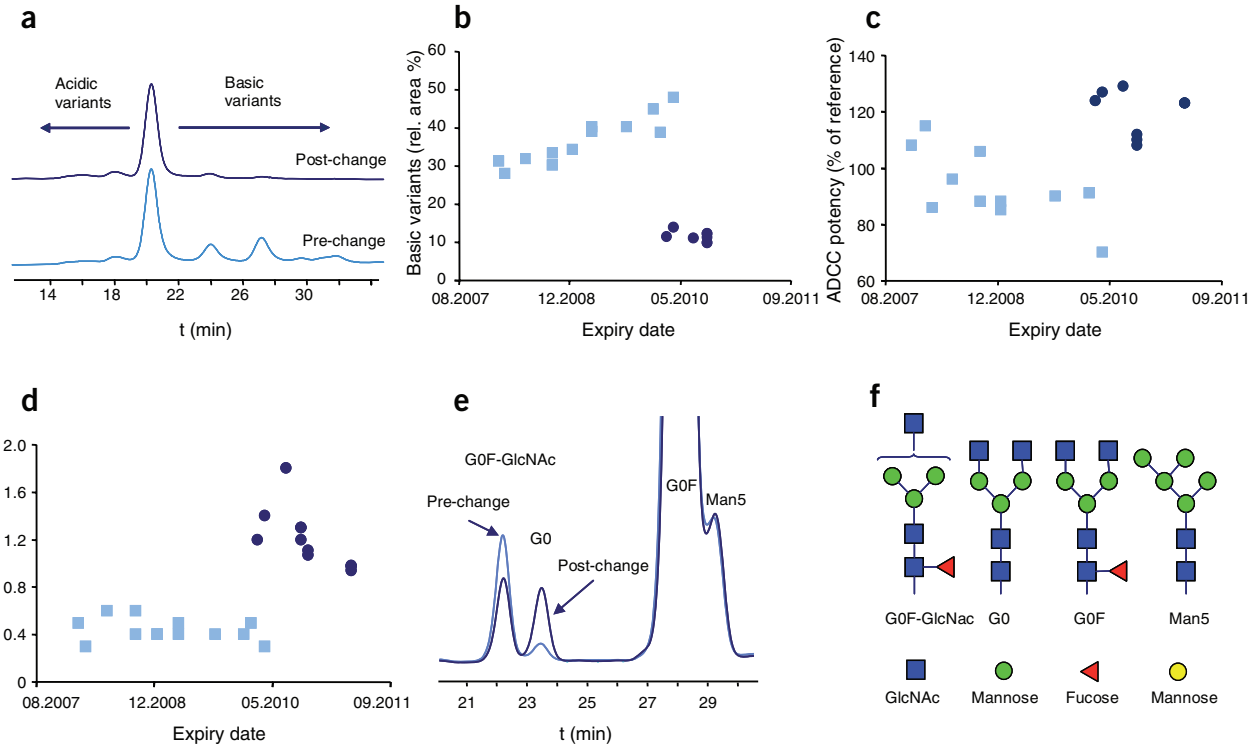

Figure 2 Comparison of the different pre- and post-change batches of Rituxan/Mabthera. (**a**) Exemplary CEX chromatograms. (**b**) Amount of basic variants of the pre-change (n = 12) and post-change (n = 6) batches as measured by CEX. (**c**) ADCC potency of the pre-change (n = 11) and post-change (n = 8) batches. (**d**) Relative amount of the G0 glycan of the pre-change (n = 13) and post-change (n = 11) batches. (**e**) Exemplary glycan mapping chromatograms. (**f**) Glycan legend.

antibodies[7,8], which most likely has no significant impact on the biological properties of the molecule. However, our finding is an indication of an alternation in the product, which might be related to a change in the manufacturing process.

Another physicochemical difference was detected in the glycan map for unfucosylated G0 glycans (**Fig. 2d,e** and **Supplementary Fig. 2b,d**). The abundance of this structure is only ~1%, but it has a substantial effect on ADCC potency, which is reflective of an essential part of the clinical mode of action. MAbs having only unfucosylated glycans are known to exert much higher ADCC potency than the fucosylated ones[9]. The abundance of unfucosylated G0 glycan in Rituxan/Mabthera increased approximately by a factor of three (**Fig. 2d**) and the measured ADCC potency also showed an increase (**Fig. 2c**), although by a factor less than 3, indicating that ADCC may depend on structural features in addition to the level of unfucosylated G0.

A third product, Enbrel, contains a dimeric fusion protein that binds tumor necrosis factor (TNF) alpha as the active ingredient. It comprises the extracellular ligand-binding domain of the human 75 kDa (P75) tumor necrosis factor receptor (TNFR2/p75) and the Fc part of a human IgG1 antibody. The protein is glycosylated containing the IgG1-specific *N*-glycosylation sites in the Fc part and multiple *O*-glycans in the receptor part[10]. We have analyzed commercial batches sourced in the European Union and the United States using glycan mapping and CEX. The data revealed a highly consistent quality profile for batches having expiry dates until the end of 2009. After this time period, batches with a second and changed quality profile appeared on the market in parallel (**Fig. 3**). Major differences were found in the glycosylation profile. The amount of variants containing the *N*-glycan G2F decreased from ~50% in the pre-change to ~30% in the post-change material (**Fig. 3b,d** and **Supplementary Fig. 3b,d**). The CEX analysis showed a change of the amount of the basic variants, which corresponds primarily to C-terminal lysine variants from 15–30% in the pre-change to 40–60% in the post-change material (**Fig. 3a,c** and **Supplementary Fig. 3a,c**). As for the other products, Aranesp and Rituxan/Mabthera, the pre- and the post-change versions of Enbrel were also marketed under the same label.

In conclusion, the data we present here reveal substantial alterations of the glycosylation profile for all tested products. Different lots of Rituxan/Mabthera and Enbrel also showed changes of the N- and C-terminal heterogeneity, and the former also showed variation in ADCC activity among batches. Because of the abruptness and the magnitude of the observed alterations, they are most probably caused by changes in the manufacturing processes. As the glycosylation profile is defined by the production cell line, growth conditions and the purification sequence, these findings may reflect changes in one or more of these components. The data indicate the magnitude of changes in quality attributes of marketed products. All tested products remained on the market with unaltered labels in the tested time frame, indicating the observed changes were predicted to not result in an altered clinical profile and are therefore acceptable by the health authorities.

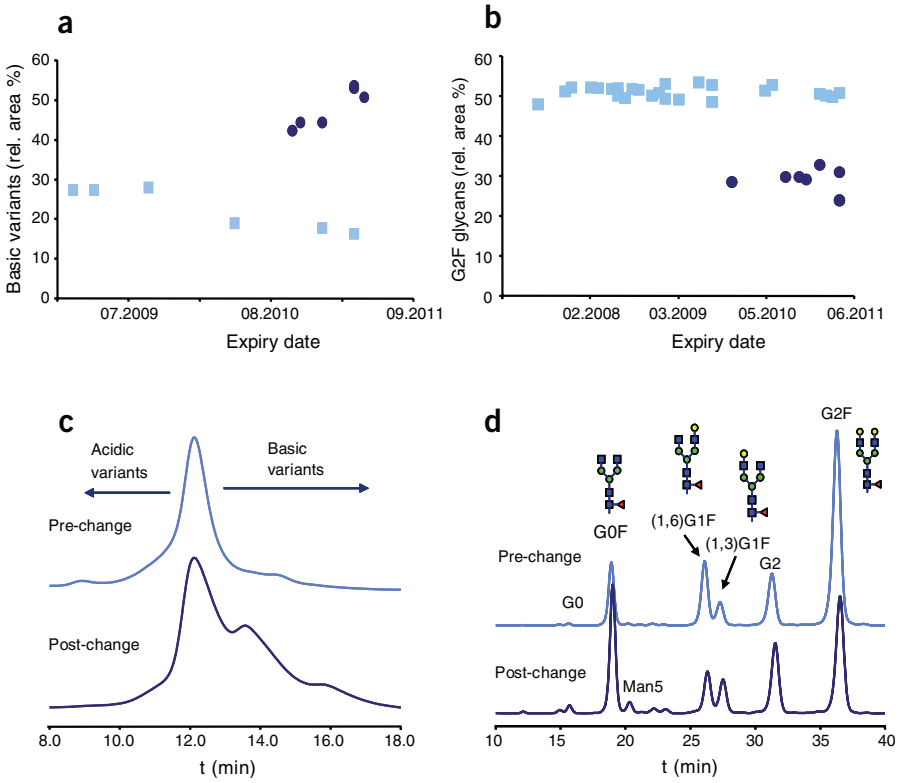

Figure 3 Comparison of the different pre- and post-change batches of Enbrel. (**a**) Relative amounts of basic variants of the pre-change ($n = 6$) and the post-change ($n = 6$) batches as measured by CEX. (**b**) Relative amount of the G2F glycan of the pre-change ($n = 25$) and the post-change ($n = 9$) batches. (**c**) Exemplary CEX chromatograms. (**d**) Exemplary glycan mapping chromatograms.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGEMENTS

NK3.3 cell line was generated by J. Kornbluth, St. Louis University School of Medicine, and the cell line was obtained by Novartis from St. Louis University, St. Louis. We would like to thank J. Windisch, M. McCamish, C. Sonderegger, M. Lang-Salchner and A. Seidl from Sandoz Biopharmaceuticals for their thorough review and support.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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