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Glycoscience – a new frontier in rational drug design

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Glycans are the most abundant and most diverse biopolymers in nature. Because of their highly specific interactions with physiological receptors, they participate in many crucial biological processes. All these processes are potential targets for therapeutic intervention, and carbohydrate-based drugs are rapidly being taken up by the modern biotechnology and pharmaceutical industry. Recent developments in the field of glycobiology have overcome the problem of glycan analysis and synthesis; and many compounds based on carbohydrates are now in various stages of clinical trials. This article presents glycoproteins in a new light, as an important biopharmaceutical target, giving an overview of their potential use as therapeutic glycoproteins and proteoglycans, inflammation blockers, cancer therapeutics and vaccines, inhibitors of pathogenic microbes, viral inhibitors and potential aids in the treatment of lysosomal diseases, neurological diseases and transplantation rejection.

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INTRODUCTION

Although structurally and functionally very diverse, glycoproteins, glycolipids, glycosyl phosphatidyl-inositol (GPI) anchors and proteoglycans, jointly called glycoconjugates, share one common feature – they all bear oligosaccharide chains (glycans) within their structure. Glycans are the most abundant and the most diverse biopolymers in nature. Due to their enormous structural diversity, oligosaccharide chains are ideal media for coding biological information. Their attachment affects many properties of glycoconjugates, such as folding, secretion, biomolecular interactions, serum lifetime and many others. Based on the highly specific interactions between glycans and their physiological receptors – lectins, glycoconjugates participate in many crucial biological processes such as cell-cell and cell-matrix interactions, adhesion, signaling, differentiation and development. Placed mainly on the outer surface of the cells, glycans may serve as identification molecules to the surrounding world (1). They mark cells and tissues as 'self' and send signals to the immune system when tissue is injured. On the other hand, tumor cells and

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viruses use glycans on their surfaces to slip past the immune system. Many pathogens use specific glycan structures of the host-cell membrane glycoconjugates to stick onto the host cells and to spread themselves to the surrounding cells. Some inflammatory reactions are triggered by carbohydrates and many diseases are associated with changes in glycan structures (2). All these processes are potential targets for therapeutic intervention and this is why carbohydrate-based drugs are increasingly being taken up by the modern biotechnology and pharmaceutical industry (3).

The basic principle of glycosylation, *i.e.*, the lack of template, makes it inherently sensitive to all changes within the cell, and glycan structures that are being produced at any moment actually mirror all relevant past events in the cell (4). Nearly a decade ago we hypothesized that these characteristics make glycosylation a very good candidate for an important role in the stress response (5). In the meantime, we have gathered a significant pool of evidence to support this hypothesis. Initially, we identified changes in lectins in livers of rats exposed to stress (6, 7), and subsequently we also identified altered glycosylation profiles both in humans exposed to war-stress (8–10), and in *in vitro* models of stress (11, 12). Galectin-3 was found to be particularly interesting since different types of stress had different effects on this lectin (13, 14). We were able to demonstrate that regulation of galectin-3 proceeds through NF-κB, which places it downstream from corticosteroids (and CRF) in the stress response (15). Finally, we were also able to demonstrate specific stress-induced changes in the activity of sialyltransferases (16). Very recently, we were also able to show that gangliosides and GPI-anchored proteins can spontaneously move between different cells (17), which might be an important signaling mechanism.

In 1990's, a series of simple carbohydrate based drugs failed clinical trails mostly because of inadequate binding constants for targeted receptors. Structural complexity of branched carbohydrate chains makes them difficult to analyze and hard to produce, but new automated methods promise a dramatic change and compounds based on complex carbohydrates are now in clinical trials for many diseases (18).

Therapeutic glycoproteins and proteoglycans

One of the most important groups of biotechnology products are therapeutic glycoproteins (19). Many important glycoprotein therapeutics have been expressed using various production vehicles, for example, cell lines, transgenic animals and plants (20). In precisely optimized glycosylation processes, proteins are bristled with sugars, which ensures that these molecules will remain in circulation long enough to achieve their purpose. These include the top-selling protein drugs erythropoietin (EPO), granulocyte macrophage-colony stimulating factor, and tissue plasminogen activator. Maybe the best-studied example is the red blood cell booster erythropoietin which interacts with a membrane--signal-transducing receptor and induces proliferation and differentiation of erythroid progenitors. The fact that recombinant erythropoietin has annual worldwide sales of nearly 5 billion US dollars is more than an indication of its importance and value in modern medicine. For over a decade, recombinant EPO has had a significant role in treating anemias caused by bone marrow suppression and end-stage renal disease (21).

EPO carries four sialylated complex-type N-glycans. *In vivo* activity of deglycosylated EPO is less than 10% of glycosylated EPO because incompletely glycosylated forms

are rapidly cleared by filtration in the kidney and through the action of Gal/GlcNAc/Man receptors in hepatocytes and macrophages (22, 23). This shows that glycans can have dramatic effects on the properties of glycoprotein-drugs, and that the control of glycosylation during their production is very important. This is why achieving proper glycosylation has become one of the major challenges in biotechnology. Indeed, the identification and cloning of relevant glycosyltransferases and the ability to modulate their expression, as well as use of appropriate cell lines or culture conditions, are important in this respect. For instance, siRNA (small interfering RNA) and knock-out strategies have been recently used to reduce core fucosylation of IgG produced by CHO cells (24, 25). There are also many other CHO cell lines (Lec and Lec mutants) with altered glycosylation, while extending the glycosyltransferases repertoire of insect cells has been used to generate transgenic Sf9 cells with mammalian-type glycosylation.

The technology that uses glycosyltransferases to add sugar nucleotides to carbohydrate chains of the glycoprotein-drugs after they have been secreted from the production cells was developed under the brand name GlycoAdvance (19). One of the best examples is the second-generation erythropoietin – darbepoetin alpha. Two extra N-linked oligosaccharide chains extend the Novel Erythropoiesis Stimulating Protein half-life three times, permitting a less frequent dosage regime (26).

Another multi-billion dollar carbohydrate drug category is heparin and its derivatives used in the treatment of thrombosis and other cardiovascular indications. Heparins are currently produced from pig intestines. Unfractioned heparin has been used for several decades as an anticoagulant. Its effects are based on the activation of antithrombin, which leads to the inhibition of thrombin and factor Xa, thus preventing the production of fibrin clots. Unfortunately, unfractioned heparin can bind to several plasma, platelet, and endothelial proteins, thereby producing a highly variable anticoagulant response. Low-molecular mass heparins are now replacing unfractioned heparins because they have better pharmacological properties and fewer secondary complications than unfractioned heparin (27). Enzymes of heparin biosynthesis are now being cloned and hence it should become possible to produce recombinant heparins as well.

Inflammation interrupters

In the late 1980s, several groups of researchers independently cloned the genes for three human selectins, carbohydrate-binding proteins (lectins) that play an important role in attracting leucocytes to injured sites in the body, thus promoting inflammation (28). These proteins are present on the surface of the endothelial cells of blood vessels when injured tissues release cytokines. In the early 1990s, it was shown that selectins recognize variants of the carbohydrate structure called Lewis x (Fig. 1), which can be found on the surface of circulating leukocytes (29). This binding slows down leukocyte circulation in the bloodstream and facilitates initiation of the inflammatory response.

Although Varki and coworkers showed that blocking L-selectin from binding to sialyl Lewis x (sLe^x) in mice reduced inflammatory response, their selectin inhibitors failed to work in animal disease models, so the project was discontinued.

Other researchers from the pharmaceutical industry have also developed an inhibitor of selectins that should work after a heart attack, stroke or tissue transplantation, but in final-stage of clinical trials data showed that there is no benefit of this drug. Now, in-

dustry researchers are trying to do better with a recombinant form of P-selectin glycoprotein ligand-1 (PSGL-1); it binds to P-selectin expressed on the endothelial cells and on the platelet cells, causing them to stick to leukocytes and create blood clots. By inhibiting these selectins they hope to prevent ischemia reperfusion injury and clotting events that can re-occlude an artery. P-selectin glycoprotein ligand-1 is a 240 kDa homodimer consisting of two 120 kDa polypeptide chains. The O-linked glycans displayed on PSGL--1 must undergo two specific post-translation modifications to enable PSGL-1 to function as a P-selectin receptor: $\alpha(1,3)$ fucosylation and $\alpha(2,3)$ sialylation (18). A new technology, called GlycoAdvance, will be used to develop an improved production system for the biopharmaceutical compound rPSGL-Ig, the P-selectin antagonist that is being developed to treat inflammation and thrombosis associated with acute coronary syndrome and reperfusion injury. It is currently in Phase II clinical trials in patients treated for heart attack and this technology is being evaluated for use in the production of rPSGL-Ig for Phase III clinical trials and commercial launch.

Based on the structures of sialyl-Le^a and sialyl-Le^x, a glycomimetic inhibitor for E--selectin was synthesized (30, 31). Smaller in size and more hydrophobic than sialyl-Le^x, it inhibits E-selectin 1,000 times better than sialyl-Le^x in an E-selectin mediated cell adhesion assay (32). Another selectin inhibitor, called TBC1269, was also successful in animal models of asthma and renal failure (33, 34) but did not attenuate asthmatic response in humans (35), possibly due to the different role of selectins in acute allergic inflammation in animal models than in human asthma or due to inadequate dosage.

Cancer therapy and vaccines

Several glycans, on both the tumor surface and host elements, have now been identified as mediating key pathophysiological events during the various steps of tumor progression, and many new therapeutic strategies are targeting these molecules (36). Selectin inhibitors can also be used in the therapy of malignant diseases. Metastasing malignant cells are able to bind to P-selectin on platelets, thus creating a kind of protective shield against the immune system cells. It was shown that heparin could bind to P-selectin on platelets and in that way prevent cancer cells from doing the same (37). Because of the problems heparin can cause in blood coagulation, it cannot be widely used as a chemotherapeutic agent, but other P-selecting inhibitors may possibly be more effective.

Inhibiting selectins is not the only way carbohydrates can contribute to the anticancer battle. They can be also used as cancer vaccines. Transformed tumor cells escape the immune system by exposing specific glycolipids and glycoproteins on their surfaces. This fact has opened a way to the use of carbohydrate chains from these glycomolecules as vaccines. Some cancer vaccines are undergoing clinical tests. One of them is a synthetic carbohydrate molecule created by Danishefsky (38, 39). First, he synthesized globo H, a complex hexasaccharide found in high numbers on the surface of many cancer cells, such as the prostate and breast cancer cells. Many cancer cell antigens, including globo H, are too small to provoke an immune response, but if the cancer specific antigen is attached to a molecule the immune system recognizes as foreign, the immune system can make antibodies against it. Those antibodies can then destroy any cancer cells left in bloodstream after the treatment of a primary tumor. The vaccine based on the globo H

antigen gave promising results when tested as an anticancer vaccine in patients with early prostate cancer. Phase I clinical trial in metastatic breast cancer patients has shown that fully synthetic globo H conjugate could be a good component of a polyvalent vaccine containing several other antigens (40). Danishefsky and Lloyd developed a similar vaccine for ovarian cancer patients (41). This vaccine is based on the carbohydrate antigen known as Lewis Y, which is present on the surface of ovarian, breast and prostate cancer cells.

Another antigen present on cancer cells and suitable for vaccine development is mucin, MUC-1. The mucins, normally expressed by secretory epithelial cells, are present in cancer cells with significant quantitative and qualitative changes in glycosylation (42). MUC-1 contains short carbohydrate chains such as Tn [GalNAc(α1-Ser/Thr)], sialyl Tn and the Thomsen-Friedrenreich antigen [Gal(β1–3)GalNac(α1-Ser/Thr)], which are not present on normal mucins (43). A conjugate of sialyl Tn with hemocyanin (immune adjuvant keyhole limpet) has been tested on breast cancer patients and in combination with cyclophosphamide gave good results (42).

Some other vaccines based on ganglioside immunogens present on certain types of cancer cells were investigated as well. Technologies for the manufacture of gangliosides for use as active pharmaceutical ingredients in cancer vaccines were developed. One of the cancer vaccine candidates has been tested on patients with melanoma. Another candidate has been developed for the treatment of a variety of other malignant diseases, including colorectal cancer, lymphoma, small cell lung cancer, sarcoma, gastric cancer and neuroblastoma (44). All these investigations showed encouraging results, but it is unlikely that cancer vaccines will be able to cure cancer on their own, but they will probably be components of a multi therapeutic approach, adjuvant therapy in early-stage diseases or combined with adjuvant chemotherapy (45).

Vaccines based on specific glycoconjugates and lectins are of scientific and commercial interest, not only against cancer, but also as anti-protozoan and anti-viral vaccines. For instance, the galactose binding lectin of the protozoan *Entamoeba histolytica* (46) and hyperglycosylated mutants of human immunodeficiency virus (HIV) type 1 monomeric gp120, as novel antigens for HIV vaccine design (47), are also targets being considered by academic and especially industrial pharmaceutical research groups.

Inhibitors of pathogenic microbes and toxins

Many infectious diseases are initiated by binding of surface lectins of pathogenic organisms to complementary surface carbohydrates of host tissues. An understanding of these interactions becomes increasingly important since microbial resistance to antibiotics becomes a more and more acute problem (48). Many potentially deadly bacteria can evade the majority of antibiotics we rely on. The best way of preventing this is to introduce new medications when the old ones no longer work**.** The idea is that, instead of looking for factors that kill bacteria or halt their reproduction, we should look for compounds that interfere with bacterial adhesion. Knowing the principle of adhesion, it can be predicted that soluble glycans or glycan mimics could be used to block the initial attachment of microbes and toxins to cell surfaces, thus preventing or suppressing the infection (49) (Fig. 1). Because many of these bacteria gain access through airways or gut, the carbohydrate-based drugs can be delivered orally or bronchially without needing to

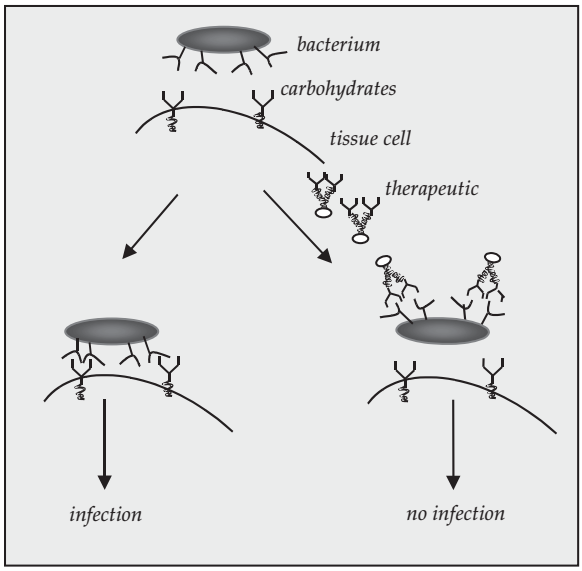

Fig. 1. Schematic representation of the interference of therapeutics with adhesion of pathogenic bacteria in order to prevent infection.

be distributed systemically. These anti-adhesion carbohydrate therapies may be considered mild, gentle and safer as compared to the present chemotherapy approaches. Carbohydrates are ideal for these interventions since they are unlikely to be toxic or immunogenic and many of those which inhibit bacterial adhesion are normal constituents of cell surfaces or body fluids, especially of human milk (50, 51). It was shown that soluble carbohydrates recognized by the bacterial lectins block the adhesion of bacteria to animal cells *in vitro.* This has also been shown in different mammals such as mice, rabbits, calves and monkeys. However, in phase II clinical trials the pentasaccharide, shown to be acting as an anti-adhesive against *Streptococcus pneumoniae* and *Hemophilus influenzae in vitro,* failed to protect young children from nasopharyngeal colonization and, thus, did not prevent the development of otitis media (50). Possible explanations for this observation include the fact that the drug was delivered by a nasal spray, that bacteria can express multiple specificities (so the inhibition may require a cocktail of oligosaccharides), and the fact that children may have different carbohydrate receptors than adults. However, recent receptor binding studies disclose a novel class of high-affinity inhibitors of the *Escherichia coli* FimH adhesion , and there is still good reason to believe that this kind of anti-microbial therapy has a bright future.

It has also been suggested that compounds affecting glycosylphosphatidylinositol (GPI) biosynthesis in the bloodstream form of *Trypanosoma brucei* should be trypanocidal (53). Thus, the GPI biosynthetic pathway was a potential drug target against African human sleeping sickness. Scientists have described cell-permeable analogues of a GPI intermediate that are toxic to this parasite but not to human cells.

Treatment of lysosomal storage diseases

There are approximately fifty known lysosomal storage diseases that afflict humans. These rare diseases are caused by mutations that result in inactive or missorted enzymes, leading to the accumulation of particular substances in lysosomes. The effective treatment for these diseases is based on the ability to compensate the missing enzyme activity. This treatment can be effective only if the enzymes are properly targeted at the lysosomes (54). A good example of successful targeting, both with recombinant and extracted enzymes, is the glucocerebrosidase replacement therapy of Gaucher's disease (55, 56). This most common lysosomal storage disorder is caused by the defective activity of acid-ß--glucosidase leading to accumulation of glucosylceramide, particulary in cells of the macrophage lineage (57). Uptake of the enzyme in replacement therapy is achieved by remodeling its oligosaccharide chains to expose core mannose residues. This modified enzyme is then taken up by mannose receptors and is delivered to lysosomes where it supplements the defective enzyme (58). A different way to treat Gaucher's disease type I patients is the »substrate reduction therapy« (SRT) that reduces cellular glycosphingolipid biosynthesis. An imino sugar *N*-butyl-deoxynojirimycin (NB-DNJ) that inhibits activity of the first enzyme in the glucosylating sphingolipid synthetic pathway was tested in clinical trials and has shown to be an effective therapy (59).

Carbohydrate-based drugs for neurological diseases

Carbohydrates have also found their use in treating neurological diseases. There are attempts to develop carbohydrate drugs for Parkinson's disease and other neurological indications (26). The development is initially focused on the modification of certain glycolipid compounds that have previously demonstrated clinical promise. Since there is no known prevention or cure for this disease and current treatments focus only on controlling the symptoms of the disease and do not retard its progression, this would be a significant improvement.

Viral deconstructors

Carbohydrates could also be used in preventing viral invasion. It seems that even a minor interference with sugars on the proteins of viral coats of hepatitis B and hepatitis C viruses can have major effects. Two drugs that are supposed to do that are both variants of natural sugars and they work by inhibiting two glycoprotein-processing enzymes in the endoplasmatic reticulum where cells add carbohydrates to newly synthesized proteins. When *N*-nonyl-deoxynojirimycin (NN-DNJ) was added to human liver cells, glycoprocessing on the hepatitis B virus that had invaded liver cells was disrupted, and the virus could not construct its critical component – M envelope protein (60, 61). *In vitro* tests showed that inhibition of 6% of cellular glycoprocessing resulted in a more than 99% reduction in the secretion of hepatitis B virus. This change is lethal for the virus but it has no effect on host cells.

There are also several carbohydrates that have promising *in vitro* biological activities against HIV (62, 63). Unfortunately, none of these molecules have yet been proven as a good entry inhibitor *in vivo*.

Transplantation rejection

When it comes to blood transfusion and transplantation, carbohydrates have the unwanted role of a barrier. Rejection is the result of hosts having a high titer of pre-existing antibodies (probably due to the prior reaction to microbial carbohydrate structures) against the carbohydrate epitopes. A similar problem is encountered when it comes to xenotransplantation (64). Using animals as organ sources seems to be a good solution for the shortage of donated human organs, but different carbohydrate patterns remain a problem. Apes and humans, contrary to other vertebrates, do not express oligosaccharide chains terminated with unfucosylated Gal(α1–3)Gal linked to glycoproteins. Since this structure is abundant in various foods, anti-αGal antibodies are present in high concentrations in human serum (65), which causes hyperacute rejection of organs transplanted from a donor species such as pigs (66). One potential way to overcome hyperacute rejection is to block anti αGal antibodies or to remove them from circulation. The pentasaccharide Gal(α1–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc has been shown to be a natural ligand for anti αGal antibodies and its synthetic equivalent is being evaluated *in vitro* (67). Another solution to this problem would be to produce transgenic pigs that express human proteins lacking αGal structures (68).

CONCLUSIONS

With the advance in the knowledge about the roles of glycoconjugates and lectins in the development of many diseases, glycobiology is increasingly coming into the focus of pharmaceutical and biotechnological companies. Cancer, angiogenesis, tissue repair, skeletal development, cardiovascular disease, microbial infection and many more disease processes involve carbohydrates. Thus, they are logical candidates for drug design. Carbohydrate based drugs appear to be safe, secure and specific. They are smaller than proteins, more easily formulated for delivery into the body and more stable. As anticipated, the demands for manufacturing therapeutic glycoconjugates will soon exceed the current capacities, so it is expected that in the near future the pharmaceutical industry will significantly increase its material and financial resources as well as intellectual efforts for the purpose of extending the knowledge about the »glyco-world«. Hopefully, they will succeed and there will be many more new »sweet« drugs in the near future.

Abbreviations and acronyms. – EPO – erythropoietin, Gal – galactose, GlcNAc – *N*-acetylglucosamine, Man – mannose, Le^x – Lewis x, PSGL-1 – P-selectin glycoprotein ligand-1, gp120 – glycoprotein 120, GPI – glycosylphosphatidylinositol, NB-DNJ – *N*-butyl-deoxynojirimycin, NN-DNJ – *N*-nonyl-deoxynojirimycin.

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S A Ž E T A K

Glikoznanost – nova smjernica u suvremenom dizajniranju lijekova

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Glikani su najrasprostranjeniji i najraznolikiji biopolimeri prisutni u prirodi. Zbog svojih visokospecifičnih interakcija s fiziološkim receptorima sudjeluju u mnogim ključnim biološkim procesima. Svi ti procesi potencijalna su meta terapeutskih intervencija pa lijekovi bazirani na ugljikohidratima nalaze svoje mjesto u suvremenoj biotehnologiji i farmaceutskoj industriji. Razvojem glikobiologije prevladan je problem sinteze i analize glikana pa su mnogi spojevi bazirani na ugljikohidratnim strukturama trenutno u različitim fazama kliničkih ispitivanja. Ovaj članak predstavlja glikoproteine u novom svjetlu, kao važne biofarmaceutske ciljeve, dajući pregled njihove potencijalne primjene kao terapijskih glikoproteina i proteoglikana, inhibitora upale, lijekova i vakcina u liječenju tumora, inhibitora patogenih mikroorganizama i virusa, te potencijalnih sredstava u liječenju lizosomskih i neuroloških bolesti te transplantacijskih reakcija.

Ključne riječi: glikoproteini, lektini, terapeutici

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nificant at the significance level $p < 0.05$. Statistical analysis was performed using the SigmaStat program, version 2.0 (Jandel Corporation, USA).

RESULTS AND DISCUSSION

Diabetic patients and control individuals were analyzed by assigning their sera to the paraoxonase phenotypes using the method of Eckerson *et al.* (23), based on the basal paraoxonase activity distribution profile (Fig. 1). Only the low activity AA phenotype was well defined at the nadir of 400 U L^{-1}, while the other two phenotypes, AB and BB, were not distinctive. Therefore, sera were classified to the paraoxonase phenotype AA and to a group comprising both the subjects with AB (heterozygous intermediate activities) and BB phenotypes (homozygous high activities).

We found that 64% of male and 64% of female control individuals belong to the AA phenotype (Table II). This is in agreement with another study in a population from Zagreb, where 60% individuals were attributed to group AA, also based on visual estimation of the basal paraoxonase activity distribution profile (25). In the diabetic group, a significantly lower percentage of male sera (45%, $p < 0.05$) and female sera (49%, $p < 0.05$) were assigned to AA phenotype. This is in agreement with the report of Ruiz *et al.* (26), the first in a series of studies on higher frequency of B allozyme and coronary heart disease in type II diabetic patients. There were no differences in paraoxonase activities between the gender- and phenotype-matched diabetic and control groups. Enzyme activities against phenylacetate were significantly higher in the sera of diabetic patients compared to the gender- and phenotype-matched control groups. Additionally, statistically signifi-

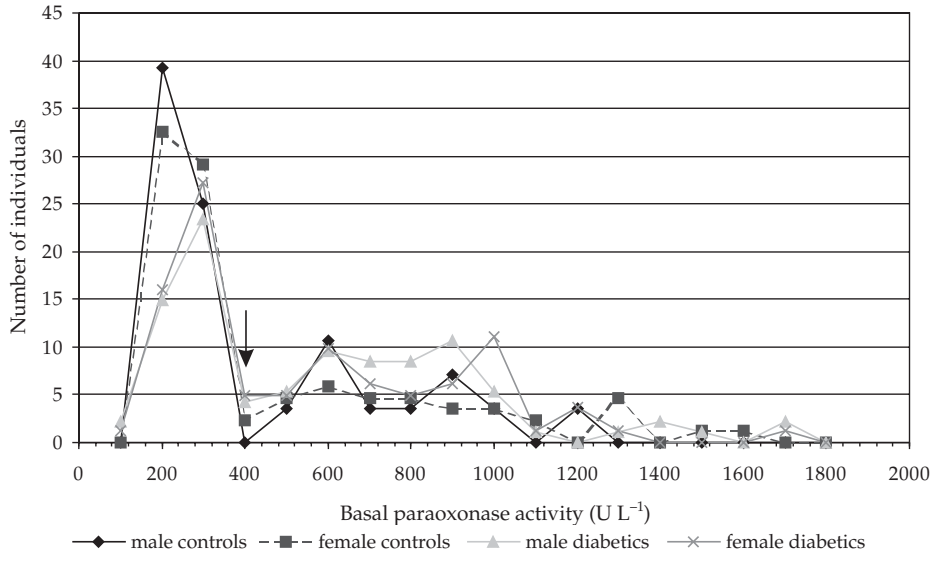

Fig. 1. Paraoxonase activity distribution in control and diabetic individuals according to gender.

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