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Severe rheumatoid arthritis prohibits the pregnancy-induced decrease in α3-fucosylation of α_1-acid glycoprotein

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Patients suffering from rheumatoid arthritis (RA) may experience a temporary reduction of disease symptoms during pregnancy. As indicated by the occurrence of RA-disease symptoms during pregnancy, three categories of patients were defined, namely, remission, relapse and unchanged. In all three categories changes in the plasma level and glycosylation of α_1-acid glycoprotein (AGP) were determined longitudinally in comparison to those occurring in pregnancy of healthy women. In healthy pregnancy, we observed: (i) a peak in the plasma concentration at week 18 and a minimum at week 30; (ii) a continuous increase in the degree of branching of the glycans during the entire pregnancy period, and (iii) a decrease in the degree of α3-fucosylation of AGP-glycans with a minimum occurring at week 25. Comparable pregnancy-induced changes in glycosylation were found for two other acute-phase proteins α_1-protease inhibitor (PI) and α_1-antichymotrypsin (ACT). Increased oestrogen levels, known to occur during pregnancy, may be one of the factors that induce these changes, because the increased branching and decreased α3-fucosylation is in agreement with our earlier findings regarding an involvement of this hormone in the regulation of acute phase protein glycosylation in oestrogen-treated males as well as females. In all three clinical categories in RA, pregnancy also induced a continuous increase in the degree of branching of the glycans of AGP. However, similar changes in concentration and fucosylation were only found during remission of the disease symptoms. In the relapse and unchanged categories in RA, the degree of fucosylation and the plasma concentration of AGP remained constant throughout pregnancy. This indicates a relationship between changes in α3-fucosylation of AGP and RA disease activity.

***Keywords*: rheumatoid arthritis, Pregnancy, α,-acid glycoprotein, α_1-anitchymotrypsin, α_1-protease inhibitor, fucosylation**

***Abbreviations*: AAL, *Aleuria aurantia* lectin; ACT, α_1-antichymotrypsin; AGP, α_1-acid glycoprotein; APP, acute-phase protein; A0, Aw and As, APP glycoforms that are non-reactive, weakly reactive, respectively, strongly reactive with AAL; CAIE, crossed affino immunoelectrophoresis; Con A, concanavalin A; C0, Cw and Cs, APP glycoforms that are non-reactive, weakly reactive, respectively, strongly reactive with ConA; HSPC, human serum protein calibrator; PI, α_1-protease inhibitor; RA, rheumatoid arthritis**

Introduction

Acute phase proteins (APPs) are proteins that are synthesized mainly by the liver. In response to inflammatory stimuli such as tissue injuries or infections, a so-called acute phase response is initiated [1], resulting in altered hepatic synthesis and glycosylation of the APPs [2, 3]. Positive and negative APPs can be distinguished, according to whether an increase or decrease in serum concentration occurs during the acute phase response. α_1-Acid glycoprotein (AGP, orosomucoid), α_1-antichymotrypsin (ACT) and α_1-protease inhibitor (PI), are such positive APPs. Several different glycoforms of the APPs can be discriminated, differing in the presence of di-/tri- and/or tetraantennary glycans, degree of α3-fucosylation and expression of the blood group

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determinant sialyl Lewis^x (sLe^x, NeuAcα2 → 3Galβ1 → 4(Fucα1 → 3)-GlcNAc-R). AGP contains five N-linked glycans [4], ACT contains four [5] and PI contains 3 N-linked glycans [6]. The degree of branching of the glycans on AGP has been shown to decrease under the influence of cytokines during acute inflammatory disorders such as septic shock [7] and intercurrent infections in systemic lupus erythematosus or rheumatoid arthritis (RA) [2, 3]. Similar results have been found for ACT and PI [2, 8–11].

Not only the degree of branching, but also the degree of α3-fucosylation of AGP has been described to change under acute inflammatory conditions such as severe burns, septic shock, febrile attacks in hyperimmunoglobulinemia D, and also in chronic inflammatory disorders such as rheumatoid arthritis [10–15]. An increase in fucosylation invariably coincides with an increased expression of the blood group determinant sLe^x on AGP [3, 7, 10, 11, 13].

Recently, we have shown that under the influence of estrogen, administered orally to male-to-female transsexuals, not only the degree of branching of AGP glycans increases, but that a decrease in the degree of α3-fucosylation is also found, leading to decreased expression of sLe^x [17]. These changes are in contrast to changes that are found during acute inflammation, where decreases in branching always coincide with increases in α3-fucosylation [3, 7, 10, 13].

Increases in degree of branching of the glycans of AGP, but also ACT, have been described during late pregnancy [8, 16]. Since physiological increases in plasma estrogen levels are known to occur during pregnancy, we have investigated in a longitudinal study whether pregnancy in healthy women also induces a decreased α3-fucosylation of AGP, and whether found changes in glycosylation and concentration of AGP also occur for two other positive APPs, ACT and PI.

Furthermore, we have included pregnant women suffering from RA before pregnancy in this study, since it has been described that often a remission of disease activity in RA is observed during pregnancy [18, 19] or with oral contraceptive use [20]. In addition, it has been established that the degree of α3-fucosylation of AGP is correlated with disease activity in RA [11, 15]. To determine whether correlations could be found between changes in glycosylation and RA disease activity during pregnancy, patient groups were divided into three clinical categories: remission, relapse and unchanged.

Materials and methods

Source of sera

Serum samples from healthy pregnant women were taken at 5, 10, 15, 18, 25, 30 and 35 weeks of gestation, and up to 4 days post partum. Between the years of 1981–1987 all pregnancies in patients with definite RA according to the American College of Rheumatology (ACR) criteria [39] from participating centers in the UK, Ireland and Holland, were entered into a cohort study investigating the effects of pregnancy on RA. A total of 61 pregnancies in 58 patients were investigated. The patients were seen every 4 weeks from enrollment into the study until up to 16 weeks post-partum. Activity of their RA was assessed clinically using the Camp Index [22]. This index is reported to have low inter and intra-observer error and entails examining each joint and scoring for pain on pressure (0–3), pain at rest (0 or 1) and synovial swelling (0–3). From the above cohort, 23 patients with moderate disease activity (four or more active joints) on admission, as assessed by the referring clinician during the previous 6 months, were selected and divided into two main groups. Patients were considered to have achieved a spontaneous clinical remission (Rem + ; n = 11) if by the third trimester the mean camp index was less than 16. Those patients not meeting this criteria [23] were assigned to the no remission group (Rem − ; n = 12), which was further sub-divided into the unchanged (n = 6) and relapse group (n = 6), if by the third trimester the mean Camp Index had remained the same or increased respectively, compared to that prior to pregnancy. For each patient we endeavored to analyze six serial serum samples from differing time points during the pregnancy: one from each of the first, second and third trimester and from the first, second and third 5 week period post-partum. All sera were kept frozen at − 80 °C during storage, a condition which does not affect the parameters investigated in this study (W. van Dijk, unpublished results).

Protein concentration

Concentrations of AGP, PI and ACT were measured in sera by radial immunodiffusion according to Mancini *et al.* [24], using monospecific polyclonal antisera (rabbit anti human acute phase protein) for precipitation (Dakopatts, Glostrup, Denmark). HSPC consisting of pooled sera from healthy blood donors was used as a standard (Dakopatts, Glostrup, Denmark). According to the manufacturers information, the concentrations of proteins in this preparation were: AGP, 0.81 ± 0.01; PI, 1.22 ± 0.01; ACT, 0.29 ± 0.01 mg ml^{-1}.

Crossed affinity immunoelectrophoresis

CAIE was performed on serum samples according to the method of Bøg-Hansen [25] with some modifications, using 2 mg ml^{-1} concanavalin A (Con A) as the diantennary-specific affinity component, or 2.5 mg ml^{-1} (with a haemagglutination titre of 1024) *Aleuria aurantia* lectin (AAL) as the fucose-specific affinity component, as described previously [11]. Con A binds the unsubstituted groups of α-linked, 2-**O**-substituted mannose residues at carbons 3, 4, and 6 with at least two interacting mannose molecules being required for the binding. As a result, Con A binds with di- but not with tri- or tetraantennary glycans [26]. AAL was isolated from fruiting bodies of the *Aleuria aurantia* mushroom as detailed earlier [13]. Although the binding

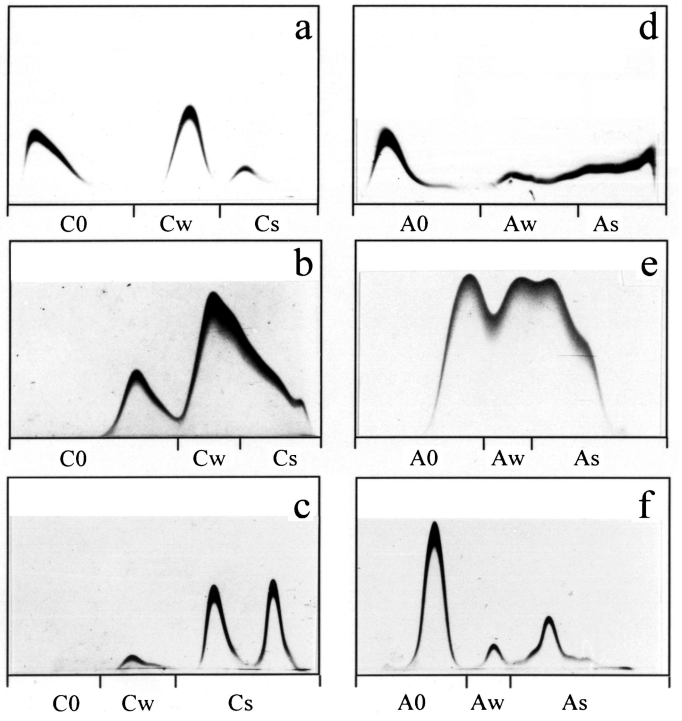

Figure 1. Reactivity of AGP (a, d), ACT (b, e) and PI (c, f) from healthy individuals (HSPC) with ConA (left panel) and AAL (right panel). Sera were subjected to CAIE as described in the Materials and Methods. Only the second dimension gels are shown. The lower right corner of each pattern corresponds to the site of application in the first dimension gel. Electrophoresis was performed from right to left in the first dimension, and from bottom to top for the second dimension. C0, A0: AGP-glycoforms non-reactive with Con A or AAL, respectively; Cw, Aw: AGP-glycoforms weakly reactive with Con A or AAL, respectively; Cs, As: AGP-glycoforms strongly reactive with ConA or AAL, respectively. No fractionation was obtained when samples were analyzed in the absence of lectin, resulting in recovery of all APP-protein at the position of C0 or A0.

specificity of AAL is not restricted to the α3-linked fucose residues, in case of AGP only α3-linked fucose is present [27]. Therefore, all retardation of AGP by AAL in CAIE can be attributed to the presence of α3-linked fucose. For PI, it is known that α6-linked fucose residues are also present [28, 29]. Briefly, CAIE was performed by applying 0.5–2.0 μl of serum to a lectin containing 7.5% polyacrylamide gel, which resulted in the fractionation of the acute phase proteins into glycoforms, reactive and non-reactive with the lectins (see Figure 1). In the second dimension, these separated glycoforms were detected by immunoelectrophoresis using the precipitating monospecific antiserum (RAH-acute phase protein-IgG). The resulting precipitation line was visualized by staining with Coomassie Brilliant Blue R250 (Sigma, St Louis, MO, USA). The areas under separated peaks of the precipitation line, indicated the relative amounts of the glycoforms (see Figure 1), which were determined using a Summagraph (ACECAD D-9000) coupled to a 386 SX PC, equipped with an area measurement program. From these areas, the relative distribution of reactive and non-reactive glycoforms were calculated.

Statistics

All values were tested for statistical significance using the Student *t* test.

Results

Effect of pregnancy on concentration

Healthy individuals

In Table 1, the variation is given in the serum concentration of the positive acute phase protein AGP during pregnancy in healthy individuals as percentages of the values obtained for standard control sera (HSPC). A maximum in concentration was observed at week 18, a minimum at week 30, and an increase post partum (> week 40). Corresponding post-partum increases were observed for two other positive APPs, ACT and PI. During pregnancy, the concentration of ACT remained at the same level or was slightly lower than HSPC values, but the concentration of PI was instead increased throughout the entire pregnancy period in accordance with the literature [30].

Patients with RA

In Table 2, the concentrations of AGP during normal healthy pregnancy and during pregnancy in patients with RA who went into remission, relapsed or remained unchanged, are given as the mean concentrations during the indicated periods of pregnancy. The changes in AGP concentration during pregnancy in patients with RA that went into remission, corresponded with those found in healthy pregnancy. As in healthy individuals, the concentration reached a maximum value between weeks 16–25, showing a further increase compared to HSPC post partum. It should be noted that the values for remission in the period 0–5 weeks are based on one data-point, which excludes statistical analysis of other values compared to this point. No significant changes in AGP concentration were found in the unchanged and relapse groups during pregnancy, although there were large variations in the values. However, the AGP concentrations in these two groups were higher than HSPC at the beginning of pregnancy and remained constant during the whole pregnancy period, showing no increase post partum.

Effect of pregnancy on the degree of branching of glycans

It is known that, following oral administration of estrogen, the degree of fucosylation of AGP and as well as the amount of diantennary glycans on AGP are decreased [17]. Therefore, we were interested in knowing if similar changes in glycosylation would occur during pregnancy,

Table 1. Concentrations and degrees of reactivity of AGP, PI and ACT with Con A and AAL relative to HSPC values in sera from healthy pregnant women.

Week	*AGP*			*ACT*			*PI*		
	conc. (%)	*C0 (%)*	*Aw + As (%)*	*conc. (%)*	*C0 (%)*	*Aw + As (%)*	*conc. (%)*	*Cw (%)*	*Aw + As* (%)
5 (n = 6)	97±22	96±11	96±11	93±11	139±37	91±12	170±20$^{\#\#}$	99±22	89±17
10 (n = 6)	103±28	103±19	96±18	91±11	92±19*	93±27	173±43$^{\#\#}$	117±27*	85±10$^{\#\#}$
15(n = 6)	96±30	139±21*,$^{\#}$	95±13	86±17$^{\#}$	126±20,$^{\#}$	91±18	191±32$^{\#\#}$	178±46**,$^{\#\#}$	85±15$^{\#}$
18 (n = 6)	115±18$^{\#}$	146±22**,$^{\#}$	81±13$^{\#}$	98±27	140±25*,$^{\#\#}$	89±17	216±19**,$^{\#\#}$	222±57**,$^{\#\#}$	76±17$^{\#\#}$
25 (n = 6)	100±20	167±27**,$^{\#}$	69±15**,$^{\#\#}$	86±16*	172±16**,$^{\#\#}$	77±14*,$^{\#\#}$	192±26$^{\#\#}$	236±66**,$^{\#\#}$	79±19$^{\#}$
30 (n = 6)	66±19*,$^{\#\#}$	169±6**,$^{\#\#}$	95±11	104±10	160±21**,$^{\#\#}$	78±12*,$^{\#\#}$	194±87$^{\#}$	192±24**,$^{\#\#}$	106±11**
35 (n = 7)	75±24$^{\#\#}$	185±7**,$^{\#\#}$	85±20	97±1	185±10**,$^{\#\#}$	89±32	264±73*,$^{\#}$	251±45**,$^{\#\#}$	107±28
40±(n = 11)	169±63*,$^{\#\#}$	151±14**,$^{\#\#}$	105±12	146±17**,$^{\#\#}$	90±6**,$^{\#\#}$	89±14	262±61*,,$^{\#\#}$	187±33**,$^{\#\#}$	120±24*,$^{\#\#}$

Values are given as the mean percentage ± SD of the HSPC values for AGP, ACT and PI, being respectively 0.81±0.08, 0.29±0.03 and 1.22±0.05 mg ml^{-1}, 41 ± 2 and 41±3% C0 and 8±0.5% Cw (PI), and 66±5, 60±3 and 39±2% Aw + As. The number of samples tested is given in brackets. HSPC, pooled control serum; C0, glycoforms of APPs non-reactive with ConA; Cw and Aw, glycoforms weakly reactive with Con A and AAL respectively; As, glycoforms strongly reactive with AAL (*cf.* Figure 1). See Materials and methods for details. * and **: significantly different from value at t = 5 weeks, $p < 0.05$, resp. $p < 0.01$. $^{\#}$ and $^{\#\#}$: significantly different from HSPC value, $p < 0.05$, resp. $p < 0.01$.

Table 2. Concentrations and degrees of reactivity of AGP with Con A and AAL relative to HSPC values in sera from healthy individuals and patients with RA,comparison between different states of disease during pregnancy.

Week	*AGP concentration (%)*				*C0 (%)*				*Aw + As (%)*			
	Healthy	*Remission*	*Relapse*	*Unchanged*	*Healthy*	*Remission*	*Relapse*	*Unchanged*	*Healthy*	*Remission*	*Relapse*	*Unchanged*
0–5	96±22(6)	67±0(1)	113±35(4)	135±47(4)¶	96±11	111±0	77±7¶	104±4¶	96±11	106±0	107±5	115±8
6–15	100±29(6)	93±28(5)	105±50(3)	137±72(5)	121±27*,$^{\#}$	107±14	79±25†	112±18	96±16	86±20$^{\#}$	95±13	94±18
16–25	108±21(6)	112±23(7)	105±33(3)	129±53(7)	157±27**,$^{\#\#}$	144±8$^{\#}$	123±7*$^{\#\S}$	126±19*$^{\#}$	76±15*$^{\#}$	75±20$^{\#}$	99±27†	94±10*†
26–39	70±22(13)**,$^{\#}$	98±22(7)¶	107±43(3)	140±33(4)¶	176±10**,$^{\#\#}$	151±20$^{\#\P}$	111±35¶§	137±23*$^{\#\P}$	90±17*$^{\#}$	73±22$^{\#}$	98±3*§	97±17*§
40 +	175±74(11)*,$^{\#}$	152±52(20)	125±26(12)¶	138±39(15)¶	152±16**,$^{\#\#}$	115±17$^{\#\P}$	97±23*¶	108±17¶	104±15	84±15$^{\#\P}$	108±9§	99±10§

Values are given as the mean percentage of HSPC values ± SD of AGP, being 0.81±0.08 mg ml^{-1}, 41±2% C0 and 66±5% Aw + As. The number of samples tested is given in brackets. For the healthy individuals, the same data as in Table 1 were used, with a new division into the indicated time periods; see Table 1 for abbreviations used. * and **: significantly different from value at t = 5 weeks, $p < 0.05$, resp.p < 0.01. $^{\#}$ and $^{\#\#}$: significantly different from HSPC value, $p < 0.05$, resp. $p < 0.01$; ¶significantly different from healthy value, $p < 0.05$. §significantly different from remission value, $p < 0.05$. †:significantly different from remission value, $p < 0.10$. For absolute HSPC values see Figure 1.

where estrogen plays a significant role, and also whether other positive APP would be affected in the same way. CAIE was used to determine the types of glycosylation: Figure 1a–c shows typical precipitation curves of AGP (1a), ACT (1b) and PI (1c) for CAIE with Con A as diantennary-specific affinity component, and Figure 1d–f shows the same for AAL as fucose-specific affinity component.

Healthy individuals

In Table 1, the changes in degree of branching of the glycans of AGP and ACT during healthy pregnancy, are represented by the changes in the %C0 relative to control values (HSPC). C0 is the fraction of glycoforms which is not retarded by Con A in CAIE, and therefore contains no diantennary glycans, but only tri- and/or tetra-antennary glycans [27]. Since a glycoform of PI without diantennary glycans does not exist (no C0, see also Figure 1c), we have used the changes in %Cw relative to HSPC-Cw, as a measure for changes in the degree of branching of PI glycans. Cw is the fraction of APPs containing only one diantennary glycan [27].

The data given in Table 1 show that the degree of branching of all three APPs increased continuously during healthy pregnancy. For AGP, C0-values increased continuously from 97% at the beginning of pregnancy (week 5), to 185% at 35 weeks of pregnancy (significantly different from value at week five and HSPC-value, $p < 0.05$), indicating that the amount of glycoforms with only tri- and/or tetraantennary glycans increased constantly during pregnancy. The same can be stated for ACT, for which an increase from 139% at week 5 to 185% at week 35 was found. For PI, the relative %Cw increased from 99% at week 5, to 251% at week 35. Post partum, all APPs showed a decrease in the degree of branching of their glycans, relative to the values towards the end of pregnancy (week 35), although only ACT reached C0-values below those found in normal healthy individuals (90% of HSPC), and AGP C0-values and PI Cw-values remained increased relative to HSPC (151% and 187%, respectively, Table 1).

Patients with RA

Essentially the same data were found for the branching of AGP glycans during pregnancy in patients with RA, irrespective of disease activity during pregnancy (Table 2). A rise in degree of branching of AGP glycans was found for all three clinical categories after 15 weeks of pregnancy, although the initial data for the relapse group at weeks 0–5, are significantly lower.

Effect of pregnancy on the degree of fucosylation of glycans

The changes in degree of fucosylation of APPs during pregnancy are represented by %(Aw + As), the sum of the glycoforms containing fucose, relative to HSPC values.

Healthy individuals

In Table 1, the changes in fucosylation for AGP, ACT and PI during pregnancy in healthy individuals, are given. Interestingly, the degree of fucosylation decreased during pregnancy, up to week 25 for all APPs studied. The values for %(Aw + As) found at week 25 for AGP (69% of HSPC), ACT (77% of HSPC) and PI (79% of HSPC) were significantly lower than the corresponding HSPC-values, and in case of AGP and ACT also than the values at week 5. After week 25, the degree of fucosylation of all three APP began to rise again, returning to HSPC-values post partum.

Patients with RA

The changes in degree of fucosylation of AGP-glycans during pregnancy in patients suffering from RA, are given in Table 2. In the healthy and remission groups, the degree of fucosylation decreased to values lower than HSPC values, reaching a minimum at approximately 25 weeks. In contrast, in the relapse and unchanged groups, the degree of fucosylation remained constant and equal to HSPC-values throughout pregnancy and post partum.

Discussion

The longitudinal set-up of the present study, in contrast to previous studies [8, 16], has enabled us to gain additional insight into the changes occurring in the regulation of the plasma concentration and glycosylation of the positive acute phase proteins AGP, PI and ACT during healthy pregnancy. Firstly, we have found that the plasma concentration of all three APPs was increased post partum over control values, but that APP-specific variations occurred during pregnancy. Thus for AGP a peak value was detected at week 18 and a minimal value at week 30, whereas the plasma concentration of PI, in accordance with the literature [30], was found to be significantly increased over control values throughout pregnancy. Secondly, with regard to changes in glycosylation, all three APPs were found to behave in the same way. They exhibited a continuous increase in the degree of branching of the glycans during the entire pregnancy period, and a slight decrease in branching post partum relative to the values during pregnancy. Furthermore, they all displayed a decrease in degree of fucosylation of the glycans during pregnancy with a minimum occurring around week 25.

With respect to the effect of pregnancy on RA, it can be concluded that the changes in concentration and glycosylation of AGP in patients with RA responding with remission of disease during pregnancy, are virtually identical to those found in healthy pregnancy. This is in sharp contrast to the patients that do not respond with remission, where changes are less pronounced or completely absent. The changes in fucosylation found for the unchanged and relapse groups in the 16–25 week period and thereafter, are significantly different from those found for the remission values. These

results are indicative of a relationship between the changes in concentration and fucosylation of AGP during pregnancy in patients with RA and the disease activities. This is in agreement with earlier observations, since we and others have shown a relationship between RA disease activity and the extent of fucosylation of AGP and for other APPs as well [2, 11, 14, 15]. This relationship has implications for the role of highly fucosylated AGP in the pathogenesis of RA. AGP is known to have several anti-inflammatory properties in which its glycosylation is involved [31, 32]. A possible role for highly fucosylated AGP expressing sLe^x in inflammation has been suggested by us, namely interference in the selectin-mediated endothelial-leukocyte adhesion involved in the inflammatory response [13]. Under the influence of pro-inflammatory cytokines, E-selectin is induced on endothelial cells, among other adhesion molecules. These same adhesion molecules have been shown to be induced on endothelial cells under the influence of estrogen [33], of which the plasma levels are known to be increased during pregnancy [34]. Taking into account that cell-mediated immunity is compromised in pregnant individuals [35], it may be suggested that an abundance of highly fucosylated AGP molecules during pregnancy could inhibit the extravasation of leukocytes, thereby immunocompromising the pregnant individual even further. In conclusion, it is feasible to assume that an increased plasma concentration of AGP molecules without sLe^x could protect the pregnant individual from further immunosuppression during pregnancy.

Interestingly, oral estrogen treatment in male-to-female transsexuals also induced an increase in branching and a decrease in fucosylation of AGP glycans [17], which is opposite to changes found during acute inflammation, where a decrease in branching and an increase in fucosylation is usually found [2, 3, 7]. It is known that, among other factors, the plasma estrogen-level increases constantly during pregnancy, and that estrogen can influence the expression of cytokine genes [34, 36–38]. Therefore, the changes in glycosylation of APP glycans such as we have found during pregnancy, may be due to a direct or indirect influence of estrogen on the liver, which is the site of synthesis of these APPs [1–3]. However, despite increasing estrogen levels in the late phase of pregnancy [34], an increase in fucosylation of the glycans of the APPs was detected after week 25. These findings indicate that besides estrogen also other unknown pregnancy-related factors seem to be involved in the regulation of the observed changes in fucosylation of the APPs. In addition, our study support earlier findings [3] that the expression of the respective hepatic glycosyltransferases involved in the changes in glycosylation of the APPs, must be regulated independently, since the pregnancy-induced changes in degree of fucosylation and branching of the glycans of the APPs occurred independently from each other.

Finally, our data with regard to the branching of the glycans confirm results found by Raynes [8] and Wells *et al.* [16], who showed increased branching of the glycans of the same APPs during late pregnancy, and a persistence of the effects of pregnancy post partum. As a result of our study, we would like to suggest that the persistence of effects post partum is counteracted by the onset of an acute inflammatory reaction due to delivery, thereby explaining the decreases in branching and increases in serum concentration we have found post partum, relative to the values found during pregnancy.

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