Genetic variants contribute to differences in response and toxicity to drugs used in autoimmune diseases: Rheumatoid arthritis and systemic lupus erythematosus

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Chapter 11

General discussion and future perspectives

RA is an autoimmune disease characterized by chronic inflammation of the synovial joints resulting in joint destruction, polyarthritis and functional disability. SLE is a chronic inflammatory disease of unknown cause that can affect virtually any organ of the body. In recent years, the use of DMARDs, anti-TNF drugs and rituximab has resulted in an improvement in the treatment of RA patients by reducing both inflammation and joint damage, and their clinical use has become widespread (1, 2). Similarly, rituximab has become a pivotal therapy in the treatment of SLE, since an aberrant B cell regulation is among the common pathogenic mechanism of these diseases (3, 4).

However, despite the use of the above-mentioned drugs, it is known that there are considerable differences in individual responses to MTX, anti-TNF and rituximab both regarding efficacy and toxicity.

The reason for this variable response is unknown, but genetic and environmental factors are thought to be implicated. Given the potential toxicities and the high cost of therapies, it would be a great improvement to be able to predict whether an individual patient will benefit from this treatment, beforehand. Knowledge about related genetic variants, mostly SNPs, may help to predict drug response or the optimal dose in the individual patient. Classically, explorative pharmacogenetic association studies are aimed at finding polymorphisms potentially useful as predictive biomarkers of drug response.

RHEUMATOID ARTHRITIS

In **chapter 2**, we reviewed the scientific literature for evidence for genetic markers for MTX-induced hepatic injury in RA treatment. Overall, we found limited evidence and a low number of studies. Such studies may be difficult due to the relative low incidence of MTX-induced hepatotoxicity. In addition, the use of different definitions of hepatotoxicity, differences in MTX dose and folic acid supplementation and the lack of replication studies hampers solid conclusions. Nevertheless, the identification of genetic predictors for MTX-induced hepatotoxicity presents an important opportunity to identify individual patients at risk for this debilitating adverse event. In general, from the published studies, *MTHFR* C677T appears to be the most promising genetic marker predicting low-dose MTX-induced hepatotoxicity (5), although because the limited power of studies to identify genetic biomarkers for hepatotoxicity, conflicting results exist limiting its clinical application.

In **chapter 3**, the association of four polymorphisms (rs1532269 and rs17301249, intronic polymorphisms mapped within *PDZD2* and *EYA4*, respectively, and rs12081765 and rs7305646 located at intergenic regions on chromosomes 1 and 12, respectively) previously

identified as being associated with anti-TNF treatment response in patients with RA was not confirmed. In addition, the combined analysis with the three previous studies included in our meta-analysis (6-8) showed only a suggestive association of one of the four polymorphisms (rs1532269) (even weaker than that reported in the study by Plant et al. (6). These findings seem to exclude effects of sufficient magnitude to be useful in predicting response to treatment. The lack of replication provided in pharmacogenetic studies could be ascribed to multiple differences between studies including ethnic background, phenotype definition or exposure to other risk factors. It is commonly impossible to identify one of them as being more relevant than the others. Genetic differences between populations are an unlikely explanation of the results, given that the allele frequencies of the four tested polymorphisms were very similar between studies. Clinical differences between the patients with RA included in the different reports are possible and difficult to exclude. In this regard, it has already been mentioned that Plant et al. (6) evaluated the response to TNFi at 6 months, whereas the two subsequent studies used the response at 14 weeks. However, this difference does not apply to our study in which evaluation at 6 months evidenced negative results.

It has been shown repeatedly that in the first study of an association, the effect is overestimated, and that there is only a modest correlation between effects in first and in subsequent studies on the same association (9-12). There is a phenomenon known as 'winner's curse' (13) or 'Jackpot effect' (14) originating in the fact that the associations with the strongest effects are inflated (10). This occurs primarily because with a small sample, a weak effect becomes significant only if the effect is overestimated. This phenomenon is aggravated by a selective reporting of the analyses, possibly biased interpretation of results and publication and other forms of bias (10, 11, 15).

It should be noted that the four SNPs studied by Plant et al. (6) showed the highest effects in the discovery cohort (which was the only one with a clear association between these four polymorphisms and the clinical response), whereas the three replication studies showed lower effect sizes (β-values less different from zero), thus supporting this possibility. Indeed, significant heterogeneity between studies was observed in the meta-analysis of three of the four analyzed genetic variants. Interestingly, this heterogeneity disappeared when the discovery cohort of Plant et al. was removed (6). Therefore, variables other than the presence of the four SNPs considered herein could have influenced the efficacy of TNFi in this cohort, accounting for its singularity. Other GWASs of responses to TNFi treatment in RA have been published (15-18). This GWAS approach represents an important step forward in the understanding of the influence of genetic variability on the efficacy of this therapy. Only one of the observed associations has been found to reach the GWAS statistical

significance level, however, and only after combination with data derived from replication studies (19). This highlights the important role of validation studies in determining the status of the remaining GWAS findings. It is to be expected that these combined efforts will produce useful insights.

This heterogeneity between studies was also seen when preparing for **chapter 5**. Several studies evaluated the hypothesis of a decreased clearance of anti-TNF drugs in RA due to *FcGR2A* and *FcGR3A* genetic polymorphisms by analyzing the effect of these SNPs on the response to different TNFα antagonists in RA with conflicting results. These disconcordant results could be explained by the small sample size, heterogeneity in the design (different anti-TNF agents), the use of different definitions of response, the different observational period and the use of different methods for genotyping. In **chapter 5**, we present the results of the first large study on the influence of *FcGR2A* and *FcGR3A* genes on treatment response in a cohort of RA patients using adalimumab as the anti-TNF drug being investigated. Our results indicate that the *FcGR2A* genotype shows a trend toward association with clinical efficacy of adalimumab defined as EULAR good response at 14 weeks. However, we did not find an association with good response or remission response for the *FcGR3A* genotype. Recently, Montes et al. (20) reported a significant association between the *FcGR2A* polymorphism and response to treatment with infliximab at 3 months, but they could not find such an association combining etanercept and adalimumab treated patients. Unfortunately, no analysis of patients treated with adalimumab or treated with etanercept could be performed separately because these two groups consisted of too small numbers of patients. In our study we were able to include 302 patients treated with adalimumab, the largest sample size for a pharmacogenetic study of adalimumab-treated patients published to date.

Previously, three papers studying the association of *FcGR3A* polymorphisms and response to anti-TNF drugs have been published (21-23). In a small study consisting of 30 RA patients, Tutuncu et al. (21) found that patients with *FcGR3A*-FF genotype had a better response to several anti-TNF drugs after 12 weeks than those carrying at least one *FcGR3A*-V allele. However, the response to therapy was not evaluated according to accepted standards such as the EULAR criteria. In contrast, Morales-Lara et al. (22) found no significant association between the *FcGR3A*-FF and good response EULAR or ACR20 criteria at 3 months in their small cohort of 41 RA patients treated with infliximab, but the genotype was associated with ACR20 response at 12 months using ACR.

Similarly, different articles have shown that the role of *FcGR* polymorphisms in response to anti-TNF drugs may be dependent on the disease as well. Several articles have studied the

association between *FcGR3A* in the response to infliximab in patients with psoriatic arthritis and ankylosing spondylitis and unexpectedly found that the high-affinity-V158 allele was associated with a better response to infliximab in patients with ankylosing spondylitis. In addition, in a recent publication (24) the presence of high-affinity alleles of *FcGR2A* and *FcGR3A* was significantly associated with a better response in the intermediated point of treatment but not at the end of the treatment in 70 PsA patients treated with different anti-TNF drugs suggesting that ADCC-mediated apoptosis of TNF-bearing cells by natural killer cells and macrophages might induce a faster clearance of milder lesions than those with higher score disease. These results suggest that the role of *FcGR* polymorphisms in response to anti-TNF drugs may be dependent on the disease as well.

In **chapter 4** it is shown that the response to anti-TNF therapy is also influenced by a polymorphism affecting the disease activity. Increased expression of IL-6 in patients carrying the *-174*C* allele would result in a poorer response to anti-TNF treatment (16, 17). The original effect on anti-TNF treatment response caused by the change in *IL-6 -174G/C* was successfully replicated in an independent population, supporting the role of this polymorphism as a genetic marker predicting anti-TNF treatment outcome. The combined analysis of our data and those previously published showed an association between this genetic variant and the clinical response to anti-TNF. IL-6 has the ability to induce an acute inflammatory reaction and, in the chronic phase, to support the activation of lymphocytes and myeloid cells, which may elevate the serum levels of IL-6, leading to increased inflammation. It may therefore be responsible for many of the systemic manifestations of RA (25). It has been shown that the neutralization of the TNF-a results in the suppression of various proinflammatory cytokines, including IL-6 (26, 27). Functional studies have reported that the *-174*C* allele is associated with higher serum levels of IL-6 (16, 17), thus suggesting that increased expression of this cytokine in patients carrying the *-174*C* allele would result in a poorer response to anti-TNF treatment. In fact, it has been shown that although both TNF-a and IL-6 are major targets of therapeutic intervention in RA, baseline serum IL-6 but not baseline TNF-a level is a potential biomarker reflecting disease activity (28). According to our data, *-174G/C* was significantly associated with a good or moderate EULAR response at 12, 18, and 24 months, but not at 6 months. Moreover, the longer the treatment period, the stronger the observed association signal was. This highlights the importance of assessing the response to long-term anti-TNF treatment. This may be the reason that an association between this polymorphism and the clinical efficacy of anti-TNF therapy has not been reported in previous pharmacogenetic studies, most of which did not evaluate the clinical response beyond 6 months of treatment (6-8, 29).

The possible interactions between two gene variants could not explain the response to anti-TNF treatment. In **chapter 5**, the high affinity *FcGR2A**H allele was associated with EULAR good response at 14 weeks in adalimumab treated of RA patients, but not with high affinity *FcGR3A*-V allele. The applied additive genetic model for *FcGR2A* and *FcGR3A* didn't show an association with EULAR good response.

SLE AND OTHER AUTOIMMUNE DISEASES

Recent studies have provided evidence that antagonizing the action of proinflammatory cytokines, including IL-6 and IL-2, may exert a therapeutic effect in autoimmune disease patients nonresponsive to other therapies. B cell depletion induced by rituximab resulted in a downregulation of proinflammatory cytokines and consequently, a decrease of the autoimmune response and re-establishment of the immunotolerance (18). The establishment of pharmacogenetic markers to predict the response to rituximab therapy becomes a pivotal requirement, given the expanding clinical use of this drug in the treatment of several autoimmune diseases. The *-174G/C* genetic variant (rs1800795), located in the *IL-6* gene promoter region, has been found associated to autoimmune diseases and involved in increased levels of IL-6 protein in serum in diverse inflammatory diseases, the GG homozygotes have circulating IL-6 concentrations approximately twice higher than those homozygous for the C allele (16). In **chapter 6** we have analyzed the association of the *-174 IL-6* promoter variation with the response to rituximab in a group of patients that presented diverse systemic autoimmune diseases. The CC genotype was borderline more frequent in non-responders as compared to those carrying GC or GG genotypes (p-value = 0.049). However, these differences were not statistically significant in SLE patients. Genotypic frequencies for CC were increased in non-responders, which correlates with the fact that patients carrying this homozygous genotype responded worse to the treatment with rituximab than those carrying GC or GG genotypes (69.2% vs. 90.2%). Fabris et al. (30) found a lower response to rituximab in RA patients that were homozygous for CC.

Their findings are in agreement with our results, both in the group of diverse systemic autoimmune diseases patients and in SLE patients analyzed separately, although, in SLE patients, the observed differences were not statistically significant, probably due to the lower statistical power of this stratified analysis. Pathogenesis of systemic autoimmune diseases involves inflammation cytokines IL-1, TNF alpha, and IL-6. Murine models in inflammatory diseases indicate that IL-6 deficiency reduces the severity of an inflammatory response (31). Recent studies have clarified evidence that antagonizing the action of

proinflammatory cytokines, including IL-6, may exert a therapeutic effect in patients nonresponsive to other therapies. Tocilizumab, a humanized antibody to the IL-6 receptor, blocks IL-6 signaling and activity and decreases levels of inflammatory markers in RA (32, 33). Previous studies reported that B cell depletion induced by rituximab resulted in a downregulation of proinflammatory cytokines, including IL-6 and, consequently, a decrease of the autoimmune response and re-establishment of the immunotolerance (18). The lower efficiency of rituximab in systemic autoimmune diseases patients carrying the CC genotype, suggests an increase in the number of refractory patients to rituximab in this group. Biological therapies different to rituximab might be had under consideration to get an adequate and more effective response in these patients. According to our data, *-174 IL-6* SNP suggests a pharmacogenetic association with the clinical response to rituximab in systemic autoimmune diseases, and the hypothesis that this variation could be a predictive value, independently of other clinical or environmental factors. Anyway, as the observed significant associations could be due to a casual finding resulting from multiple comparisons, larger replication studies are needed and still planned by our group to confirm present results. Currently, there are very few data about genetic markers of prognosis that may be used in the future to facilitate treatment decisions. We herein provide preliminary evidence of a possible new genetic marker, the CC homozygosis of the *-174 IL-6* promoter polymorphism, as a predictor of nonresponse to rituximab in autoimmune diseases.

In **chapter 4**, the *-174*G* allele was significantly associated with a good or moderate EULAR response at 12, 18 and 24 months in an independent cohort of Spanish RA patients treated with anti-TNF therapy. A meta-analysis combining these data with the results from a previous study (34) confirmed this association. In **chapter 6**, The *-174 IL-6 CC* genotype was significantly increased in non-responders with respect to responders in several autoimmune disease patients treated with rituximab. Therefore, in some way, the *-174 IL-6*G* allele could be a genetic marker of response to rituximab in different autoimmune diseases.

Rituximab is recognized and bound to the surface of NK cells and macrophages through the FcGR, triggering ADCC immune system mechanism, essential for the activity of rituximab to deplete B cells. *FcGR3A* is expressed by immune effector cells and shows specific affinity for IgG monoclonal antibodies, such as rituximab. The importance of *FcGR3A* in the response to rituximab has been shown in studies where mice lacking *FcGR3* presented a decrease in the response to this drug (35). In **chapter 7**, we have analyzed the association of the *FcGR3A*-158F/V polymorphism with the response to rituximab in patients with autoimmune diseases. Genotypic frequencies for this SNP were similar to those described previously for several patients with autoimmune diseases in Caucasian populations (36-38). It is remarkable that frequencies were elevated for V carriers in responders, which correlates

with the fact that patients carrying the V allele at this position presented a better response to the treatment with the drug than those with homozygous FF genotype. Functional studies have demonstrated that the 158V allele is correlated with a better biological response to rituximab in autoimmunity. Anolik et al. (39) showed that in patients with SLE carrying the high-affinity V allele (FV or VV), rituximab was more effective in depleting peripheral B cells than in those homozygous for the low-affinity FF. Recently, the *FcGR3A*-158F/V SNP has been associated with the clinical response to rituximab in RA. This study conducted in 111 patients found that the V allele carriage was significantly associated with a higher response rate (91% of responder vs. 70%; p = 0.006, OR = 4.6, 95% CI 1.5–13.6) (40). The findings in SLE and RA are in line with our results that showed a better response to rituximab in patients with autoimmune diseases that carried the V allele (FV or VV) than in patients with homozygous FF. Additionally, based on the previous association observed in patients with SLE and on the fact that this was the largest group, we analyzed separately patients with SLE. We found a similar pattern, and patients carrying the V allele showed a better response to rituximab treatment, although it did not reach statistical significance (p = 0.08). Finally, we examined the group of patients with no SLE to establish whether this association is shared by different autoimmune disorders. As in the case of patients with SLE, we observed a similar effect, but this association did not reach statistical significance either (p = 0.08). This suggests that the influence of the *158F/V* polymorphism in the therapeutic response to rituximab is common to various autoimmune diseases; however, the reduced numbers involved in these stratified analysis leads to poor statistical power, and therefore the conclusions are provisional.

It should be noted that copy number variation (CNV) has been shown to be present in the *FcGR3A* gene (42-44). The presence of common CNVs can cause false SNP genotyping results that can lead to fail the Hardy–Weinberg equilibrium (HWE) and may blur the association of the studied SNPs with disease susceptibility. In our study, the genotypic frequencies were significantly different from those predicted by HWE, but only in the group of patients with SLE. This may be due to existence of an association between the *158F/V* polymorphism and this disease (41). In fact, in our cohort of healthy controls (previously published genotypic data), genotype frequencies for this SNP were in the HWE (38). Moreover, the frequency of CNV has been reported to vary significantly in different ethnic populations, which can result in contradictory findings, but in this case, frequencies observed in patients were similar to those previously described, and the results reported to date are fairly consistent. Previous findings showed that patients carrying the V allele in *FcGR3A*-158F/V increased expression of CD16 in NK cells (45). A correlation between the number of cell surface CD16 receptors and the enhancing of the ADCC activity mediated by NK cells was found. These

observations would explain the better response to rituximab observed in patients with systemic autoimmune diseases carrying the V allele and would highlight the importance of the ADCC mechanism for clearance of B cells by rituximab in autoimmune diseases. In summary, our results together with previous findings (39, 40) suggest that *FcGR3A* plays an important role in response to rituximab in patients with systemic autoimmune diseases and support the hypothesis that the *158F/V* variant could be used as a potential predictor of those patients who will respond better to treatment with rituximab.

In **chapter 7**, rituximab was more effective in V allele carriers than in homozygous FF in RA patients. However, in **chapter 5**, no significant associations were found for the *FcGR3A* polymorphism and response to adalimumab in RA patients, and the combined influence of high-affinity alleles (*FcGR2A*-H and *FcGR3A*-V) showed no association between the number of high-affinity alleles and EULAR good response neither for remission. These conflicting results regarding the role of *FcGR3A* on the response to different drugs (adalimumab and rituximab) in RA patients could have a biological explanation. On the one hand, patients with high affinity allele (V) are more effective in depleting peripheral B cells and have better response to rituximab (47). On the other hand, patients with low affinity allele (F) may have decreased FcGR-mediated drug clearance of adalimumab and then a better response to this drug. This could mean a first step toward personalized medicine in RA and to choose the drug by the *FcGR3A* genotype.

In healthy subjects, stratification according to the *IL2–IL21* region polymorphism (rs6822844) revealed significant differences in circulating interleukin-2 with the lowest levels in GG genotype (19). In **chapter 8**, SLE patients homozygous for rs6822844 G allele show a better clinical response to rituximab at month 6 than patients with GT genotype. On the contrary, no association was evident in the group of non-SLE patients. It could be speculated that this lack of association was a consequence of a lower statistical power in the latter analysis. However, it should be noted that no effect size was suggested in this case (i.e. OR = 1) and, in addition, a reduction of the statistical significance of the association was observed when the non-SLE patients were meta-analyzed with those showing SLE (which increases the statistical power). Taken together, our data suggest that the influence of the *IL2/IL21* rs6822844 polymorphism in the therapeutic response to rituximab is specific of the SLE condition. Although the mechanism of action of rituximab remains unclear, accumulating data suggest that ADCC may play a dominant role (48). ADCC is mediated through immune effector cells, mainly NK cells, via expression of an activating receptor for the Fc portion of IgG antibodies (FcGR). The majority of human NK cells are CD16 positive (FCcRIII) and express the intermediate affinity interleukin-2 receptor. It has been described that intermediate doses of IL2 are capable of expanding CD16 positive NK

cells and activating cytotoxic effector functions, including ADCC activity (49-53). Several studies have demonstrated that this ability of IL2 to promote NK cell expansion and cytotoxicity influences the efficiency of rituximab treatment and correlates with the clinical response (54-59). Furthermore, the relationship between IL2 and the efficacy of rituximab is supported by the fact that soluble interleukin-2 receptor is used as a prognostic factor in patients with lymphoma receiving rituximab (60-64). An alteration of the function of B cells is a key factor contributing to SLE pathophysiology; however, some clinical trials with rituximab in this disease have failed to show efficacy. Murine models of SLE based on antibody mediated cellular depletion evidenced that this lack of efficacy can be explained by a defect in macrophage and neutrophil IgG-dependent phagocytosis induced by serum IgG (65). In this context, the role of IL2 promoting rituximab-mediated ADCC could become more critical in the efficacy of rituximab in lupus than in other autoimmune diseases in which this drug acts through all its mechanisms. The *FCGR3A-158* polymorphism is currently shown to enhance rituximab mediated ADCC and improve clinical response to this drug (45). Similarly, rs6822844 variant could affect the cytotoxic activity of NK cells and, therefore, the efficacy of rituximab in SLE condition; although to date no functional studies analyzing this issue have been published. In conclusion, we show for a first time that *IL2–IL21* rs6822844 G/T polymorphism influences the clinical efficacy of rituximab in SLE patients. The replication of this association in independent studies could enable the potential use of this variant as a pharmacogenetic marker.

In **chapter 8** the results indicate that SLE patients homozygous for rs6822844 G allele at the *IL2–IL21* region show a better clinical response to rituximab at month 6 than patients with GT genotype. On the contrary, no association was evident in the group of non-SLE patients. Interestingly, these findings show conflicting conclusions with the results obtained in **chapter 6** where the allele associated previously with lower level of IL6 were associated with worse response to rituximab.

FUTURE PERSPECTIVES

It is well known that personalized medicine is a tool that allows predicting the response or toxicity to drugs before the administration. This approach is very well accepted in some clinical areas, such as oncology, psychiatry, and is also starting in cardiology. Probably this is due to the high level of evidence of the association between genetic polymorphisms and the clinical outcome which led to the development of PGx guidelines in these areas. However, in other areas such as autoimmune diseases, among which we highlight AR and LES, at present it has not been possible to find validated genetic markers that predict

the response to drugs and thus can be used in daily clinical practice. In this case, it has been difficult to transfer knowledge of the effect of genetic polymorphisms into specific recommendations because the low evidence of the association or even disagreement between different studies.

Regulators are often confronted with challenges involved in translating data from pharmacogenomic studies into clinically relevant and meaningful product information, starting with the level of scientific evidence required to justify the inclusion of PGx data in the product information (66). For developing new drugs there is a guideline published by European Medicines Agency (EMA) which provides a framework on where it is recommended that pharmacogenetics should be implemented in the drug development process (66, 67). For authorized drugs, such as MTX, anti-TNF drugs and RTX used in RA and SLE, the guideline for the use of pharmacogenomic methodologies in the pharmacovigilance evaluation of medicinal products should be followed by researchers in order to find biomarkers associated with the response or toxicity of the drugs (68). With both guidelines, EMA intends further to enable the potential of PGx during drug development and surveillance and to gain insight into the associated scientific challenges and discusses potential solutions. The guidelines are expected to improve genomic data-informed drug development and clinical experience, thereby promoting understanding of interindividual drug response variations and, consequently, provide guidance towards more personalized treatments in the interest of the patient and public.

This thesis reflects the need to do more studies to find genetic markers that are associated with the response to drugs used in RA and SLE. The steps to follow would be the following.

Some limitations of these research studies in autoimmune diseases are that most of them use measures, including DAS-28, American College of Rheumatology, or EULAR response criteria, which include subjective measures of disease and are known to have a placebo effect (69) and they have not taken into account one of the reason that explain that patients with RA which continue with the active disease, or relapses, even during current biological therapy is the immunogenicity associated to these drugs (70).

There are different exploratory approaches providing different levels of evidence. On one side of the spectrum non-randomized (cohort, case-control or single arm) studies are performed and on the other side of the spectrum randomised controlled studies (RCTs -prospective or retrospective evaluation) are executed. The search for genetic biomarkers can be done without a hypothesis using GWAS approaches. Typically, GWAS is a search strategy rather than specific developmental design. GWAS have revolutionized genetic research as they allow the discovery of multiple gene variants with individually small effects.

The advantage of GWAS is that they eliminate the need to choose, a priori, candidate genes or variants. GWAS are highly suitable to identify genetic variants contributing to complex phenotypes such as drug response or drug- induced toxicity. The GWAS approach enables novel and less obvious genetic markers to be identified, particularly for genetic variation affecting drug pharmacodynamics, which is more complex and often less well understood than pharmacokinetics. While very interesting and affordable, GWAS also suffer from limitations. In this thesis we have shown that biomarkers found using non-RCT, including cohort studies and GWAS could not be replicated and validated in other independent studies, probably because the different definition of outcomes, the low sample size, and lack sufficient rigor to establish the predictive value of the biomarkers and to quantify its sensitivity and specificity.

Novel studies that overcome these limitations are necessary to find biomarkers which predict the response or toxicity to drugs used in RA and LES. And, after that, probably it´s necessary to conduct a clinical trial where preliminary information regarding the value of a predictive biomarker is based on published literature or from early studies within a development programme.

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