BIOMARKERS IN DRUG HYPERSENSITIVITY

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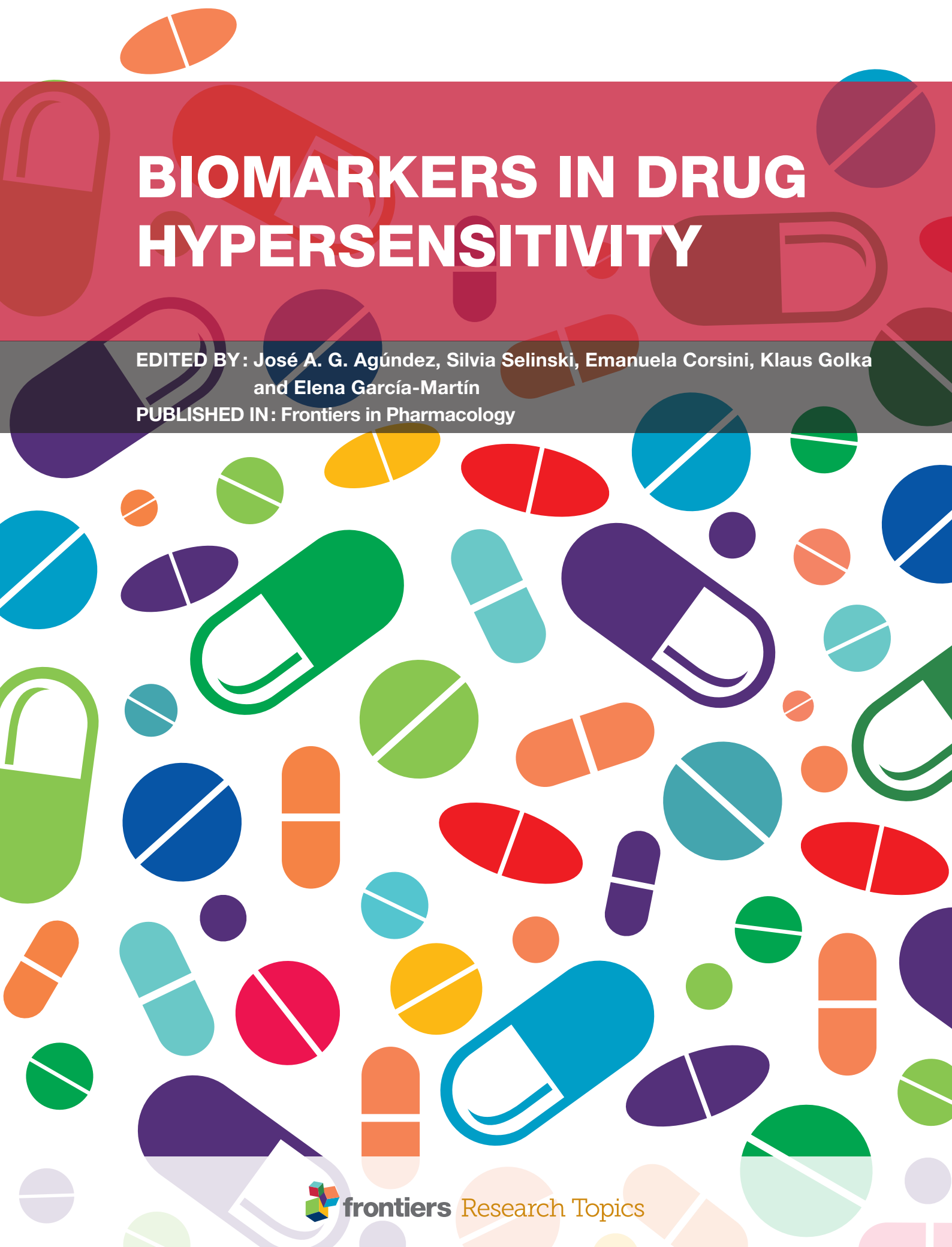

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BIOMARKERS IN DRUG HYPERSENSITIVITY

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Biomarkers, especially those based on pharmacogenomics testing, have proved to be extremely useful for type A adverse drug reactions. Clinical practice guidelines based on biomarker testing are presently being developed and updated for type A adverse drug reactions. In contrast, little attention has been paid to the potential use of biomarkers in type B adverse reactions, characterized by the occurrence of reactions not directly related to the pharmacological properties of the drug. Drug-induced hypersensitivity belongs to those type B reactions.

Drug-induced hypersensitivity reactions involve complex mechanisms that include, among others, the metabolic activation and haptenization of drug metabolites. Hence, factors that influence the pharmacokinetics of drug and metabolites may contribute to the development of some drug-induced hypersensitivity reactions. This implies that processes such as ADME (absorption, distribution, metabolism and excretion) that are typically involved in type A adverse drug reactions, may have a role in hypersensitivity reactions too. In addition to metabolic activation, several signal transduction pathways participate and modulate the development and the clinical presentation of drug hypersensitivity.

The diverse mechanisms underlying such drug-hypersensitivity reactions lead to four major groups of reactions according to the Gell and Coombs classification: immediate, cytotoxic, immune complex and delayed. The enormous complexity of drug-hypersensitivity reactions is a consequence of the variety of mechanisms involved, which may be related, among others, to drug metabolism, generation of antigenic signals, stimulation and maturation of dendritic cells, presentation of haptens and mechanisms of cytotoxicity. In addition, a plethora of possible clinical presentations exists, including urticaria, angioedema, anaphylaxis, cytopenias, nephritis, serum sickness, vasculitis, contact dermatitis, drug rash, eosinophilia and systemic symptoms, Stevens–Johnson syndrome, toxic epidermal necrolysis and acute generalized exanthematous pustulosis. The rapid progress in the field in recent years indicates that the combination of several disciplines is essential to understand the mechanisms involved in this particular, and not completely understood, type of adverse drug reactions.

The objective of this Research Topic is to present insights obtained from both basic and clinical scientists, which may include studies related to the identification, validation, refinement and clinical implementation of biomarkers for drug-induced hypersensitivity. The Topic aims to include recent findings related, but not limited to, potential phenomic, genomic, proteomic, metabolomic and signal transduction biomarkers. These biomarkers could eventually be used in clinical practice and/or these might contribute, as a proof of concept, to our understanding of the complex events leading to drug hypersensitivity reactions. In addition the Topic will cover recent developments and methodological advances in the diagnosis, prevention and therapeutic management of drug-induced hypersensitivity.

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EDITORIAL
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Editorial: Biomarkers in Drug Hypersensitivity

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Keywords: ADRs, drug hypersensitivity, drug allergy, HLA genes, DILI

Editorial on the Research Topic

Biomarkers in Drug Hypersensitivity

This special issue on biomarkers in drug hypersensitivity consists of 11 articles focusing on recent advancements related to this exciting field. Biomarkers, especially those based on pharmacogenomics testing, have proved to be extremely useful for type A adverse drug reactions (ADRs). Clinical practice guidelines based on biomarker testing are presently being developed and updated for type A ADRs (see, for instance the Clinical Pharmacogenetics Implementation Consortium website https://cpicpgx.org/guidelines/).

The World Health Organization defines ADRs as any noxious, unintended, and undesired effect of a drug that occurs at doses used for prevention, diagnosis, or treatment. Major ADR categories include type A—predictable reactions (about 80% of all ADRs), and type B—unpredictable, reactions. Where predictable reactions are usually dose dependent, related to the known pharmacologic actions of the drug, and occur in otherwise healthy subjects, unpredictable reactions are generally dose independent, unrelated to the pharmacologic actions of the drugs, and occur only in susceptible subjects. Compared to type A, type B ADRs are extremely complex and these include drug intolerance, drug idiosyncrasy, drug allergy, and pseudo-allergic reactions.

Böhm and Cascorbi elegantly summarized the different reaction types, mechanisms and known biomarkers for type B ADRs. Among these, the HLA-B alleles are highly relevant for delayed T-cell mediated reactions with abacavir (Martin et al., 2014) and carbamazepine (Leckband et al., 2013). In another article included in this Research Topic Sukasem et al. confirmed the relevance of HLA-B in the occurrence of adverse reactions (e.g., Stevens-Johnson syndrome and toxic epidermal necrolysis) secondary to the use of allopurinol in Thai patients. A previously published CPIC guideline contraindicated the use of allopurinol for carriers of HLA-B*5801 (Hershfield et al., 2013), although it should be borne in mind that the Thai population displays an unusually high frequency for carriers of such variant alleles and therefore, it is crucial to measure the strength of the association in this population in order to gain more ground on the clinical implementation of preemptive pharmacogenomics tests for HLA-B alleles. In spite of the utility of HLA testing, clinical implementation is hampered due to its technical complexity and because of the potential source of heterogeneity related to the use of diverse genotyping procedures (revised in Martin et al., 2012). The contribution by Chua and Ng in this Research Topic analyzes the potential of an additional procedure for HLA testing based on nanopore sequencing mechanisms, seeking a rapid and useful tool for the detection of genetic markers for drug hypersensitivity. They concluded that the procedure is promising, although there is still room for improvement.

Besides genetic biomarkers, clinical and analytical biomarkers provide crucial information. A careful assessment of clinical phenotypes, for instance, is essential to improve the accuracy of

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biomarkers as predictors of disease evolution and/or therapy response. Pérez-Alzate et al. identified an unusual clinical presentation among patients who were selective responders to paracetamol or a single NSAID, which usually present with urticaria and/or angioedema and anaphylaxis: A subgroup of selective responders presented with rhinitis and/or asthma with no skin manifestation. These findings suggest the occurrence of a new clinical phenotype for selective responders to NSAIDs. A particularly severe phenotype of drug-induced hypersensitivity is drug-induced liver injury. Robles-Díaz et al. summarized current knowledge on biomarkers related to diagnosis, phenotypes, clinical course and prognosis. Among these, mechanistic-based biomarkers such as the proteins High-mobility group box 1 and Keratin-18, or the micro-RNA miR-122 hold great promise.

Drug-induced hypersensitivity is often related to metabolic activation and haptenization of drug metabolites. Hence, factors that influence the pharmacokinetics of drug and metabolites may contribute to the development of some drug-induced hypersensitivity reactions. This implies that processes such as biotransformation and excretion, which are typically involved in type A adverse drug reactions, may have a role in hypersensitivity reactions too. Nuin et al. demonstrated that the active trifusal metabolite 2-hydroxy-4-trifluoromethylbenzoic acid, which causes photoallergy, is covalently bound to a protein model after photoactivation.

Other clinical presentations of drug-induced hypersensitivity reactions correspond to non-allergic mechanisms, usually associated with the release of inflammatory transmitters. Of these, eicosanoids play a prominent role in inflammation and are thought to be involved in cross hypersensitivity to NSAIDs. In this regard, it has been shown that some genetic variants of the arachidonic acid pathway influence the risk of developing such cross-hypersensitivity reactions (Cornejo-Garcia et al., 2012). Cornejo-García et al. analyzed the genetic variability of prostaglandin and leukotriene receptors, seeking for genetic biomarkers which alone, or combined with polymorphisms of the genes coding for the cyclooxygenase enzymes (Agundez et al., 2014, 2015), may help in the understanding of the mechanisms underlying cross-hypersensitivity to NSAIDs.

Several signal transduction pathways participate and modulate the development and the clinical presentation of drug hypersensitivity once the reaction is triggered. One of these depends on the interplay of IgE response and the consequent release of mediators. Amo et al. studied the genetic variability of the high-affinity IgE receptor (Fcε RI) and the variability in genes coding for enzymes involved in histamine homeostasis in patients with selective hypersensitivity to NSAIDs. They concluded that polymorphisms in the diamine oxidase (DAO) gene that have functional consequences (Ayuso et al., 2007) are involved in the clinical presentation of these selective hypersensitivity reactions, as has been reported previously in patients with cross hypersensitivity to NSAIDs (Agundez et al., 2012). Sánchez-Gómez et al. reviewed the role of enzymes involved in the generation of danger or co-stimulatory signals, such as GSTP1-1 and aldose reductase, in drug hypersensitivity. These enzymes are important regulators of the balance of inflammatory mediators, they participate in allergic processes, they can metabolize drugs and they are covalently modified by drugs, thus indicating a high potential for these enzymes in future research on mechanisms underlying drug hypersensitivity.

Two papers in this research topic focused on *in vitro* models. Galbiati et al. analyzed the potential of the use of THP-1 cell lines and interleukin-8 production together with CD86 and CD54 expression for pre-clinical immune safety evaluation studies. The proposed *in vitro* method benefits from a rationalistic approach with the idea that allergenic drugs share with chemical allergens common mechanisms of cell activation. In addition, they described the experimental conditions and markers to identify drug sensitizers, also assessing the state of the art of *in vitro* models to assess the allergenic potential of drugs. Steiner et al. reviewed the literature on a different model used in clinical studies, the Basophil Activation Test (BAT). After analyzing the potential of this procedure in hypersensitivity to beta-lactams, quinolones, neuromuscular blocking agents, contrast media, chemotherapeutics, and NSAIDs, among other drugs, they concluded that BAT constitutes a safe, complement of *in vivo* tests in immediate drug hypersensitivity reactions.

We would like to thank all the contributors whose valuable work has helped us to present wide-ranged aspects in this field.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Pharmacogenetics and Predictive Testing of Drug Hypersensitivity Reactions

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Adverse drug reactions adverse drug reaction (ADR) occur in approximately 17% of patients. Avoiding ADR is thus mandatory from both an ethical and an economic point of view. Whereas, pharmacogenetics changes of the pharmacokinetics may contribute to the explanation of some type A reactions, strong relationships of genetic markers has also been shown for drug hypersensitivity belonging to type B reactions. We present the classifications of ADR, discuss genetic influences and focus on delayed-onset hypersensitivity reactions, i.e., drug-induced liver injury, drug-induced agranulocytosis, and severe cutaneous ADR. A guidance how to read and interpret the contingency table is provided as well as an algorithm whether and how a test for a pharmacogenetic biomarker should be conducted.

Keywords: adverse drug reactions (ADRs), drug hypersensitivity reactions, drug-induced agranulocytosis (DIA), drug-induced liver injury (DILI), drug-induced severe cutaneous adverse reactions (SCARs)

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INTRODUCTION

Apart from their intended principal therapeutic use, drugs action is always related to the risk of ADRs. ADR are an important cause of morbidity and mortality. It is estimated that 3.6% of all hospital admissions are due to an ADR and that 17% of all in-patients develop ADR, an estimated 0.5% of all ADR is lethal (Bouvy et al., 2015). The mean costs of a single ADR event in Germany has been calculated as 2,743 EUR (Meier et al., 2015). An U.S. American study reports costs from 1,439 USD to 13,462 USD (Alagoz et al., 2016). Avoiding ADR is thus mandatory from both an ethical and an economic point of view. We present the classifications of ADR, discuss genetic influences with focus on delayed-onset hypersensitivity reactions, i.e., DILI, DIA and SCAR, and present an algorithm when and how to test for relevant pharmacogenomic biomarkers.

Taxonomy of Adverse Drug Reactions (ADRs)

Adverse drug reaction are divided into types A and B ADR (**Figure 1**). Type A ADR, the so-called "pharmacological ADR," are caused (i) by a change of dosage and/or pharmacokinetics and consequently of its pharmaco- or toxicodynamic action or (ii) solely by a change in the target

Abbreviations: ACEi, angiotensin-converting-enzyme-inhibitor; ADR, adverse drug reaction; AERD, aspirin-exacerbated respiratory disease; AIU, aspirin-induced urticaria; ASA, acetylsalicylic acid; CPIC, Clinical Pharmacogenetics Implementation Consortium; CysLT, cysteinyl leukotriene; DIA, drug-induced agranulocytosis; DILI, drug-induced liver injury; DRESSs, drug rash with eosinophilia and systemic symptoms; HLA, human leukocyte antigen; LTs, leukotrienes; MCH, major histocompatibility complex; NAT, arylamine *N*-acetyltransferase; NNS, number needed to screen; NPV, negative predictive value; NSAID, non-steroidal anti-inflammatory drug; PAF, platelet-activating factor; PGs, prostaglandines; PPV, positive predictive value; SCAR, severe cutaneous ADR; SJS, Steven Johnson's syndrome; TEN, toxic epidermal necrolysis; USAN, U.S. Adopted (Drug) Name; USAN, aspirin.

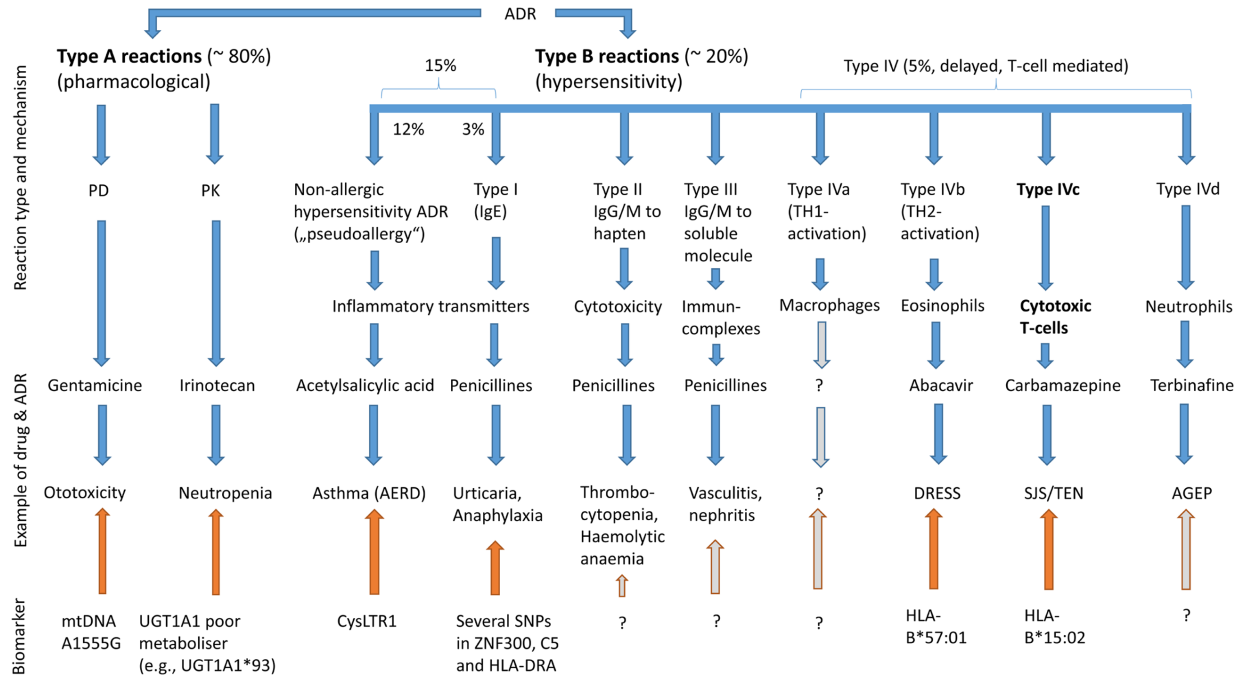

FIGURE 1 | Overview of different ADR types, examples for drugs and reactions and influencing biomarkers or patients' conditions.

structure leading to different affinity of the drug to the target and/or a different agonist-directed trafficking at the (target-) receptor. In contrast, type B ADR, drug hypersensitivity ADR, are caused by allergic or non-allergic mechanisms involving the immune system and/or mediators such as histamine (**Figure 2**). Type A were estimated to account for approximately 80% of ADR occurring in clinical practice (Borda et al., 1968). However, this figure has undoubtedly changed over the last 50 years due to differences in drug prescriptions, pharmacovigilance activities and a better understanding and thus demarcation of type B ADR. 34 years later, maybe owing to these advances in medicine, type A were reported to account for 91% of all ADR (Mjorndal et al., 2002).

In the past, it was postulated that type A ADR are usually a feature of the drug property and thus predictable, while type B ADR are strongly dependent on the genetic features of the host. Pharmacogenetic polymorphisms are now known to aggravate certain type A ADR (cf. descriptions of AERD and red-man-syndrome below). Type B ADR appeared to be non-predictable and dose-independent. However, dose-dependency has been shown for some hypersensitivity reactions (Rive et al., 2013). Rising knowledge of genetic polymorphisms of the immune system have helped to predict at least some type B ADR by applying genotyping (Rive et al., 2013).

Drug Hypersensitivity Reactions (type B ADR, Idiosyncrasy)

Depending on the mechanism of activation of the immune systems, most type B ADR (~75%) can be classified as either non-allergic hypersensitivity ADR (formerly called "pseudoallergic"), i.e., direct effect on mast cells causing histamine release, or as type I reaction according to Gell and Coombs, i.e., IgE-mediated histamine release. Type IV reactions, i.e., T-cell-mediated delayed hypersensitivity reactions, are less common (~25%). Types II and III reactions are uncommon among drug hypersensitivity reactions.

Besides these immune reactions types I to IV, a direct pharmacologic action on immune receptors ("p-i concept") of T-cells without prior presentation of the drug via MHCs (coded by HLA) has been proposed (Posadas and Pichler, 2007). Finally, some drugs are thought to alter the functioning of the immune system, e.g., alpha-methyldopa can induce the production of drug-independent autoimmune antibodies (Pierce and Nester, 2011), and statins potentiate the shifting of T-helper 1 to T-helper 2 immune responses (Suchak et al., 2007).

Examples, Clinical Manifestation, and Pharmacogenetics

While type A ADR are usually a feature of the drug, drug hypersensitivity reactions are strongly dependent on the genetic features of the host. However, pharmacogenetic markers have been described for both types of ADR.

Type A ADR

Type A ADR depend on the toxico- or pharmacodynamic action of the drug and are thus diverse. E.g., aminoglycosides are ototoxic. However, this ototoxicity can be greatly enhanced by a polymorphism in the mitochondrial DNA coding for a 12S-ribosome vital for mitochondrial protein biosynthesis resulting in complete deafness during

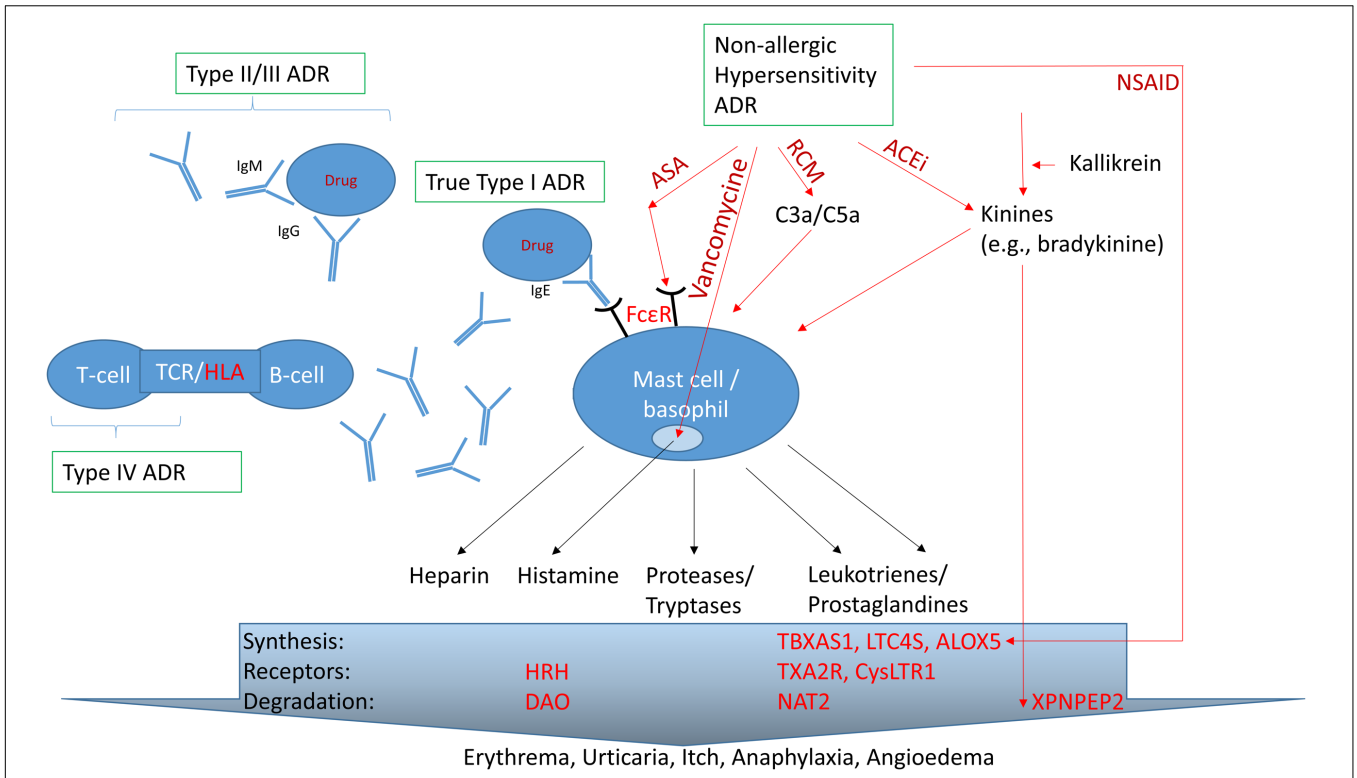

FIGURE 2 | Stimuli for degranulation of mast cells and basophils and interplay/overlap with type II-IV reactions. Beside the canonical IgE-mediated true allergic pathway, activation of the complement system and the direct interaction with IgE-receptor can lead to degranulation. Changes in the the metabolism and signaling of various arachidonic acid-derivatives, e.g., cysteinyl leukotrienes, or in the histaminergic system, as well as changes to the kallikrein-kinine-system are believed to aggravate any reactions. Light red: proteins/genes involved in hypersensitivity with known genetic associations; Dark red: example of drugs leading to primarily non-allergic hypersensitivity ADR. ACEi, angiotensin-converting-enzyme inhibitor; ALOX5, 5′-lipoxygenase; ASA, acetylsalicylic acid (USAN: aspirin); C3a/C5a, activated components 3 and 5 of the complement system; DAO, diaminooxidase; FcεR, IgE-receptor; HLA, human leukocyte antigen; HRH, histamine receptor; LTC4S, cysteinyl leukotriene synthetase; NAT, *N*-acetyl transferase; NSAID, non-steroidal anti-inflammatory drugs; RCM, radio contrast media; TBXAS1, thromboxane synthetase; TCR, T-cell receptor; TXA2R, thromboxane receptor; XPNPEP2, aminopeptidase P.

aminoglycoside treatment (Estivill et al., 1998; Usami et al., 1998). Varying activities of drug metabolizing enzymes are the main cause of type A ADR. Classic examples for such pharmacokinetic variants is the poor-metabolizer phenotype of the drug metabolizing enzyme UDP-glucuronosyl-transferase (UGT) 1A1 which results in increased risk of neuropenia during high dose irinotecane therapy (Innocenti et al., 2009) or of cytochrome P450 2D6 causing an elevated risk of extrapyramidal symptoms while treatment with the neuroleptic haloperidol (Brockmoller et al., 2002).

Immediate Reactions: Type I and Non-allergic Hypersensitivity ADR

Mast cells and basophils can be stimulated to release inflammatory agents like histamine, heparin, leukotrienes, prostaglandins, cytokines, proteases, and PAF. If the stimulus derives from an IgE-antigen-complex it is considered a true type I allergic reaction. However, non-IgE-mediated responses are common and comprise approximately 77% of all reactions of this type (Demoly et al., 1999). For some drugs, both mechanisms apply (Canto et al., 2009).

After degranulation of mast cells and basophils, the resulting type I or non-allergic hypersensivitiy ADR mainly manifest in the skin as itch, urticaria, and erythema due to the pro-inflammatory substances released. Acute severe reactions, called anaphylaxia, involve the cardio-vascular system and the airways, i.e., swelling and edema of pharynx, larynx and bronchi with possible subsequent asphyxia. Anaphylaxia is seen more frequently with immediate hypersensitivity reactions than other types.

Non-allergic hypersensitivity ADR ("pseudoallergy")

There are several pathways for non-IgE-mediated mast cell/basophil degranulation (**Figure 2**).

Non-steroidal anti-inflammatory drug are very commonly used drugs that are frequently involved in hypersensitivity ADR in some individuals. They are reported to be the leading cause of hypersensitivity ADR (Dona et al., 2012). ASA, USAN or NSAID-exacerbated respiratory disease (AERD or NERD, respectively) and ASA-induced urticarial (AIU) were known to affect primarily individuals with allergic rhinitis and polyposis nasi after application of an NSAID. This phenotype is also commonly referred to as Samter's triad. The last decades shed

light on various genetic markers associated with AERD/AIU, e.g., DAO (Agundez et al., 2012) and histamine-receptors (Ayuso et al., 2013). Other markers like IgE-receptors (FcεR) and alterations in synthases, receptors and degrading enzymes of CysLT and thromboxanes are nicely reviewed by (Park et al., 2013; Gomez et al., 2015). The phenotype "nasal polyposis" is associated with certain HLA genotypes (Molnar-Gabor et al., 2000).

However, predictive testing for AERD/AIU appears to date not feasible due to the multitude of possible biomarkers and their relatively weak associations: E.g., recalculating the frequency data on CysLTR1 haplotypes and AERD (Kim et al., 2006) suggests that at least approximately 300 patients need to be genetically tested to avoid one incident. Cf. below (see section 'The Output') for more showcase calculations and points to consider for predictive testing.

Brisk displacement of histamine from mast cells/basophils can clinically present as red-man-syndrome which is seen after rapid intravenous exposure to a variety of drugs, e.g., vancomycine, ciprofloxacine, and amphotericine B. The red-man-syndrome after application of vancomycine was linked to a missense polymorphism in the diaminooxidase (DAO) gene at c.995C > A (Myers et al., 2012). DAO is needed for efficient degradation of histamine. Defects thus cause or aggravate histamine-dependent ADR.

Mastocytosis is a pathological condition leading to large amounts of histamine being released to a variety of stimuli, including commonly used drugs like NSAIDs. While mastocytosis is poorly understood, an association with a missense variant in c-Kit gene (c.2468A > T) which encodes a tyrosine kinase receptor in stem cells is known (Nagata et al., 1995; Akin, 2006). Mutated c-Kit leads to constitutive activation of affected immune cells.

In addition to antibiotics and NSAID, other commonly used drugs or substances which can lead to non-allergic hypersensitivity ADR are radio contrast media (e.g., gadolinium, iopromid), local anesthetics (e.g., bupivacaine), opioids (e.g., morphine), curare-derivatives (e.g., rocuronium), preservatives (e.g., benzoate) and coloring agents (e.g., yellow-orange S). It appears extremely variable which of these substances actually trigger a hypersensitivity ADR in an individual susceptible patient.

Angiotensin-converting-enzyme inhibitors inhibit bradykinine degradation as off-target effect. Polymorphisms in a kinine degrading enzyme (aminopeptidase P, XPNPEP2) are thought to contribute to angioedema (Cilia La Corte et al., 2011; Mahmoudpour et al., 2013). Bradykinine is believed to worsen inflammatory responses. There are two case report of fatalities caused by allopurinol hypersensitivity possibly aggravated by concomitant captopril or enalapril, respectively (Pennell et al., 1984; Ahmad, 1995). Based on these reports, the combination of ACEi and allopurinol is considered not recommended. However, considering that this combination is extremely common and that virtually no further fatalities were reported, the mechanistic idea that ACEi will exacerbate every hypersensitivity reaction needs to be questioned.

True type I immediate ADR

Recently, various polymorphisms in several genes have been linked to penicillin-induced immediate hypersensitivity reactions (Gueant et al., 2015). Quite surprisingly, HLA genes appear to be involved, although HLA gene products are not prominently involved in IgE-signaling to mast cells and basophils. On the other hand, both production and specificity of IgE appear to correlate with certain HLA genes (Marsh and Bias, 1977; Young et al., 1995).

Penicillines and cephalosporins are listed in the WHO Model List of Essential Medicines and prescribed world-wide. Furthermore, after NSAID, beta-lactam antibiotics are reported to be the leading cause of hypersensitivity ADR (Dona et al., 2012) and the most frequent cause for true allergies (Blanca et al., 2009). Due to the high exposure rate and the intrinsic high risk, allergic reactions are occurring frequently.

Type II and Type III

Type II and type III reactions are less commonly observed. Penicillines are known to form haptens on blood cells which are subsequently targeted by IgG and IgM antibodies causing thrombocytopenia or hemolytic anemia (type II). If betalactame antibiotics a such as pencillines are bound by IgG or IgM in the bloodstream, immune complexes form and cause intra-vascular immune reactions, e.g., vasculitis, or damage the glomeruli, e.g., glomerulonephritis (type III). To our best knowledge, there is currently no data on genetic associations to such types II and III reactions.

Type IV

Type IV ADR may lead to symptomatic or asymptomatic internal manifestations include, among others, agranulocytosis (DIA), hepatitis (DILI), nephritis (DIRI), pneumonitis and myositis. Fever and lymphadenopathies are possible. Type IV ADR can also damage the skin SCAR, e.g., DRESSs, destruction of ∼10% of the skin SJS and destruction of greater extend, the so called TEN. There is a certain overlap of DRESS, SJS, and TEN concerning the dermatological features.

Type IV ADR are strongly linked with a plethora of HLA-genes residing on chromosome 6. HLA-A, HLA-B, and HLA-C encode proteins that form a MHC I-receptor on various cell types for presentation of intracellular peptides to the immune system. HLA-DP, HLA-DM, HLA-DO, HLA-DQ, and HLA-DR are proteins for T-cell interaction, e.g., MHC II-receptors and other related proteins, consisting of alpha- and beta-chains encoded by separate genes. A single HLA-gene can be further specified, e.g., HLA-B*44:02:01:02S for a HLA-B gene of allele group 44 and allele 02. The other descriptors specify synonymous changes in the coding region, changes in the non-coding region and changes in expression. Traditional serology-based HLA typing can usually only detect the allele group of the HLA protein and not the other subtle differences below this level.

Examples of hypersensitivity-conferring HLAs and type IV ADR include abacavir + HLA-B*15:02 causing DRESS (Martin et al., 2012, 2014), carbamazepine + HLA-B*31:01 causing

SJS/TEN (McCormack et al., 2011) and flucloxacillin + HLA-B*57:01 causing DILI (Daly et al., 2009). Other related genes include Transporter Antigen Processing (TAP1/2), MHC-class I related chain A/B (MICA/MICB) and hemochromatosis gene (High Fe, HFE). Data on T-cell receptors genes (TCRs) and killer cell immunoglobulin-like receptors (KIR) is scarce, but might prove relevant.

Datasources

Several databases exist nowadays to summarize the various findings (**Table 1**).

Overlapping of Different Reaction Types

Drugs can elicit hypersensitivity ADR by several non-allergic and allergic mechanisms at once, e.g., penicillines can induce immediate and delayed ADR (Blanca et al., 2009), and vancomycin can lead to both IgE-mediated and non-allergic hypersensitivity ADR (Polk et al., 1993).

To date, the different type IV reactions are not easily separated, i.e., type IVc reactions appear to be a dominant mechanism that is also occurring during types IVa, IVb, or IVd reactions (Posadas and Pichler, 2007).

Depending on the HLA-genotype, the risk agranulocytosis mediated by the antipsychotic clozapine could be significantly increased (Goldstein et al., 2014). However, a second mechanism was proposed before hypothesizing that clozapin would be metabolized to highly reactive nitrenium ions that deplete the ATP and glutathione-content of neutrophils and would ultimately lead to neutropenia (Tesfa et al., 2009).

Conditions Other than Genetics Influencing Type B ADR

The general state of the immune system may influence the occurrence or non-occurrence of ADR. E.g., vancomycin-related red-man-syndrome is very frequently seen (up to 90%) in non-infected, healthy patients, e.g., those receiving prophylactic treatment, but much less (3.7–47%) in those suffering from infections (Sahai et al., 1990; Sivagnanam and Deleu, 2003). Vice versa, reactions to ampicilline (notably, not amoxicilline) are much more common in EBV-infected patients (Haverkos et al., 1991; Chew and Goenka, 2016) and reactions to sulfonamides occur predominately in HIV-infected patients (Jaffe et al., 1983).The mechanism for sulfonamide-induced hypersensitivity in HIV-patients was attributed to a decrease of cellular glutathione (Rieder et al., 1995).

TABLE 1 | Databases of genotypes and associated ADR.

Name of database	Access
The HLA Adverse Drug Reaction Database	http://www.allelefrequencies.net/hla-adr/default.asp
LiverTox Database	http://livertox.nih.gov/
HLADR (Du et al., 2015)	http://pgx.fudan.edu.cn/hladr/

DELAYED-ONSET DRUG HYPERSENSITIVITY REACTIONS

Common Features of Drug Hypersensitivity Reactions

It can be speculated whether the clinical manifestation of a drug hypersensitivity reactions is connected to specific genetic alterations. E.g., is there a common pool of polymorphisms leading to either DILI, DIA or SCAR? Existing data do unfortunately deny the existence of such a simple genetic constellation.

Example for One Drug – One Genotype – Several Outcomes

Allopurinol causes SJS/TEN in Han Chinese bearing HLA-B*58:01. At the same time, in the same population, it can cause DRESS.

Example for One Drug – Several Genotypes – One Outcome

Vice versa to the allopurinol example, nevirapine causes DRESS. It appears that the most significant HLA genotype for this reaction heavily depends on the population: HLA-B, HLA-C, and HAL-DR-loci have been associated.

Example for Several Drugs – One Genotype – One Outcome

Finally, phenytoin, phenobarbital, and carbamazepine can elicit SJS/TEN in HLA-B*15:02 positive patients. At least this last riddle can be explained by considering the common chemical features of the three drugs (see below for details).

Drug-Induced Livery Injury

Flucloxacilline and amoxicilline/clavulanic acid are powerful first-line antibiotics which may cause DILI (Daly et al., 2009). Due to their wide-spread usage and inherent ability to cause DILI, it is vital to further understand the mechanism. Unfortunately for testing purposes, the numbers needed to screen are high and routine testing is (currently) not feasible. This in part due to the rareness of DILI which is estimated to occur in about 14 of 100,000 inhabitants per year (Bell and Chalasani, 2009). This comes unexpected when taking into account that the liver is a metabolically highly active organ, able to produce a variety of bioactivated and thus putatively immunogenic compounds from the parent drug. However, the liver is considered a tolerogenic environment, i.e., there are mostly no or locally restricted immune reactions in the liver (Crispe, 2003).

Amoxicilline/clavulanate has been linked to DILI in patients with HLA-DRB1*15:01 and recently with HLA-A*30:02, HLA-B*18:01 and the complex genotype HLA-DRB1*15:01-DQB1*06:02 (Stephens et al., 2013). Interestingly, the detected populations at risk also differed in age and speed of reaction onset.

Drug-Induced Agranulocytosis

Several drugs can elicit a life-threatening destruction of blood cells. E.g., the antipsychotic clozapine is commonly

(0.8% of drug users) causing neutropenia (less than 1500 neutrophil granulocytes/μl) or agranulocytosis (less than 500 granulocytes/μl). Other triggering drugs for DIA comprise thyreostatics like methimazole and the analgesic metamizole (USAN: dipyrone). Pharmacogenetic associations are known for methimazole and HLA-DRB1*08:03:2 (Tamai et al., 1996), for metamizole and various HLA genotypes (Vlahov et al., 1996) and for clozapine and a complex HLA genotype in Jewish subjects (Lieberman et al., 1990) for more than two decades.

Since clozapine appears to be the most effective antipsychotic drug available, there is a need to increase our understanding of its safe or putatively unsafe usage. The initial findings for clozapine were subsequently refined and applied to the general population (Dettling et al., 2001). Various polymorphisms leading to DIA after clozapine exposure have been identified, e.g., a complex genotype consisting of HLA-DRB5-DRB4, HLA-C-B-DRB5 (Dettling et al., 2007), HLA-DQB1 (Athanasiou et al., 2011), HLA-DQB1 (126Q) and HLA-B (158T) (Goldstein et al., 2014). The latter two studies deserve additional attention since the association was identified to be due to single amino acid changes at a defined position in the HLA gene product rather than to alleles described before. This is similar to the incidental finding that a single amino acid change in HLA-A, HLA-B, and HLA-C at position 152 might explain altered susceptibility of T-cells to drugs and lead to DILI (Stephens et al., 2013). Amino acids exchanges at this position alter the antigen binding pocket E of the MHC I receptor and possibly the interaction with T-cells. These findings suggest that a single amino acid change and not a serology-derived typing of HLA may provide a better prediction of the observed hypersensitivity reaction because of the mechanistical explanation inferred by the change in the binding pocket.

Severe Cutaneous ADR

Next to the liver, the skin, including the mucosa, is involved in bioactivation of drugs. Additionally, since the skin is the barrier that protects our body from the environment, it is rich of immune cells. Due to this combination of a huge amount of putatively immunogenic compounds, an abundancy of immune cells and the constant pre-sensitization of the dermal immune cells due to their contact with pathogens, the skin is a prime location for the manifestation of immune reactions. It has been proposed that the increased reactivity of EBV- or HIV-infected patients to aminopenicillines might be due to a lower activation threshold of T-cells (Posadas and Pichler, 2007). Differential pathways of activation have been show for flucloxacillin (Yaseen et al., 2015).

Severe cutaneous ADR encompass DRESS and SJS/TEN. Commonly used substances which can cause SCAR are abacavir, lamotrigine, and carbamazepine (Pirmohamed et al., 2015). The latter has an aromatic moiety and is grouped together with phenobarbital and phenytoin to the aromatic anticonvulsants. These aromatic anticonvulsants were associated with various HLA-A and HLAB variants. Interestingly, a hypersensitivity reaction to the non-aromatic drug lamotrigine was recently also associated to HLA-B*15:02 and SJS in a Han Chinese population (Man et al., 2007; Zeng et al., 2015).

The finding that abacavir is very likely to induce DRESS in HLA-B*57:01 positive patients led to a guideline of the CPIC which recommends testing because of the severity of the reaction and the high risk in absence of genetic prescreening (Martin et al., 2012). It is estimated that 6% in the general population are carriers of HLA-B*57:01 and that around 50% of carrier will develop DRESS. The recommendation is thus classified as "strong." However, it was argued that 50% of positively tested individuals will be denied an effective treatment option (Martin et al., 2012). Therefore, CPIC reviews guidelines on a regular basis. Recently, the unchanged recommendation was confirmed (Martin et al., 2014).

Table 2 summarizes all affected drugs and clinical manifestations of late-onset hypersensitivity reactions.

ISSUES IN DATA ACQUISITION AND INTERPRETATION

The Input

As nicely illustrated by the abacavir example, any improvement of signal detection and risk calculations requires correct assessment of the event (Phillips et al., 2011). Physicians in clinics tend

TABLE 2 | Manifestations of late-onset hypersensitivity reactions and commonly affected drugs.

Affected organ	Clinical manifestation	Drugs involved
Liver	Anorexia	Flucloxacilline
	Fatigue	Amoxicilline
	Nausea	
	Abdominal pain	
	Jaundice/itching	
	Blood clotting disorders	
	Blood tests: elevated liver function tests (ALAT, ASAT)	
Granulocytes	Agranulocytosis:	Metamizole (Dipyrone)
	Sudden fever	Clozapine
	Sore throat	Carbimazol/ Thiamazol/Methimazole
	Infections (urinary tract, pneumonia)	
	Sepsis	
	Blood tests: low leukocyte counts	
Skin	DRESS:	Allopurinol
	Fever	Abacavir
	Edema (face)	
	Exanthema	
	Lymphadenopathies	
	Blood tests: eosinophilia, thrombocytopenia, anemia	
	SJS/TEN:	Lamotrigine
	severe necrosis of the skin	Carbamazepine
		Phenobarbital
		Phenytoin

sometimes to misdiagnose the reaction, focus on only one aspect, fail to conduct further investigations that would strengthen or invalidate the finding, or fail to document the event appropriately (Thien, 2006). The solution that worked for the abacavir-findings was to conduct two tests, both a clinical assessment using a structured query form and a skin patch test to confirm involvement of the immune system (Phillips et al., 2011).

Starting with the thalidomide tragedy, the powerful pharmacovigilance system was installed in most countries. The strength of this system is the huge data pool on adverse events like ADR encompassing millions of cases world-wide. A major drawback is the paucity of information routinely entered into this systems (Böhm et al., 2016). Genetic information is usually missing and the patients mentioned in the individual safety reports cannot be recontacted for further investigations. It is thus only possible to generate hypotheses which need further evaluation.

The Throughput

To increase the collection of cases and their quality, interested parties have formed their own collaborative research teams, e.g., RegiSCAR for severe cutaneous reactions (Phillips et al., 2011), the Berlin Case–Control Surveillance Study group (e.g., Huber et al., 2015) and the International CIA Consortium for DIA or DILIGEN, iDILIC, DILIN, and others focusing on DILI (Nicoletti et al., 2016). These networks allow standardized collection of patient and event data as well as genetic material, and possibly re-identification of patients for further data or material sampling.

Of note, such a network requires tremendous resources if the drug-event-combination is rare. E.g., finding the association of flupirtine and HLA-DRB1*16:01-DQB1*05:02 causing DILI took more than 10 years of careful preparation and data collection (Nicoletti et al., 2016). The Berlin Case-Control Surveillance Study group collected data for 10 years (Huber et al., 2015). The increasing availability of electronic health records, the ongoing deployment of biobanks and the advances regarding the accompanying ethical, legal, technical, and social challenges (Strech et al., 2016) might deliver a global new data source for research on drug hypersensitivity reactions.

The Output

All data essentially boils down to a 2x2 contingency table of one biomarker and the occurrence of the ADR. Several statistical measurements can be derived from this: *p*-values (e.g., derived from Chi squared with Yates' correction), odds ratios, sensitivity/specificity, PPV, NPV as well as NNS.

It is sometimes desirable to combine several polymorphisms to a complex genotype or, analogously, several drugs to one drug class or several reactions to a syndrome. Grouping of individual findings will enhance the usefulness of the data for constructing novel detection techniques and to reach statistical and clinical significance (Böhm et al., 2016). E.g., genotyping of individual polymorphisms in cases of clozapine-induced agranulocytosis revealed no findings, whereas grouping polymorphisms to complex genotypes revealed three associations (Dettling et al., 2007). Another example is the 75% cross-reactivity of carbamazepine, phenytoine and phenobarbital and possibly lamotrigine on SJS/TEN. Arene oxide is a common moiety of metabolites of the first three anticonvulsant drugs and is considered to be the immunologic active substance. Grouping these drugs might help to strengthen signals for certain polymorphisms.

Traditional statistical approaches are not suited for analyses of small numbers. The fewer cases are reported, the wider the 95%-confidence interval becomes. The work of iDILIC shows that it is possible to find a convincing signal with just six cases (Nicoletti et al., 2016). However, in the future Bayesian approaches are expected to replace traditional frequentists' methods for such small numbers of cases (Yum et al., 2014). Bayesian statistics is increasingly used in early clinical trials in order to efficiently screen for signals where numbers are low.

Each of the statistical measurements given above should be used for different purposes:

(i) **Statistical significance:** the *p*-value marks the feasibility for further calculations of this drug-biomarker-reaction-association.
(ii) **Technical significance:** sensitivity/specificity are useful for test validation only. They have no direct value for the patient or the health system. Of course, the higher these values are, the better the following resulting measurements.
(iii) **Clinical significance:** PPD and NPD reflect the personal risk for an individual patient. E.g., if a drug hypersensitivity reaction is extremely rare, a positive test for a biomarker does not automatically suggest a high predictive value. PPD and NPD will guide which further clinical investigations should be conducted or which treatment options are feasible.
(iv) **Economic significance:** the NNS will be used by the stakeholders of the heath system. The NNS allows to estimate the how many patients need to be screened to prevent one case of ADR. Depending on the costs of the screening procedure and the severity of the ADR, different cut-off values for NNS will be used. Time will show whether and to what extend pharmacogenetic tests will decrease the overall treatment costs for prediction of drug hypersensitivity reactions. Current evaluations estimate a plain decrease of costs for the health system (Alagoz et al., 2016).

Table 3 shows several examples where statistical, technical, clinical and economic significance differ.

The example of the pooled data (dataset #3) as extracted from the HLADR database shows that combining data does not substantially improve PPV, NPV, or NNS if the individual datasets are of sufficient quality (Mallal et al., 2002; Martin et al., 2004).

A common problem is the acquisition of data due to the low numbers of cases and matching controls: often, not all cells of the 2x2 contingency table contain a count. Zero counts are problematic for the calculation of measurements of disproportionality. There are several ways to cope with this situation: A mathematical tool is the usage of Haldane's modification for Odds Ratios. By adding 0.5 to every cell, zero counts are eliminated. Another option is carefully matching other

TABLE 3 | Showcases of associations of drug – biomarker – event and derived statistical measurements.

#	Drug-biomarker-event	BE	Be	bE	be	*p*-value	Sensitivity/specificity	Prevalence or incidence of event	PPV/NPV	NNS	Datasource
1	Abacavir – HLA-B*57:01 – DRESS/AHSS	14	4	4	163	<0.01	77.8/97.6%	8%*	77.8/97.6%	16.58*	FD1 HLADR (Mallal et al., 2002)
2	Abacavir – HLA-B*57:01 – DRESS/AHSS	17	4	1	226	<0.01	94.4/98.3%	8%*	78.9/99.4%	15.53*	FD4 HLADR (Martin et al., 2004)
3	Abacavir – HLA-B*57:01 – DRESS/AHSS	31	8	5	389	<0.01	86.1/97.9%	8%*	79.5/98.7%	16.25*	Pooled FD1+FD4 (Mallal et al., 2002; Martin et al., 2004)
4	Abacavir – complex genotype HLA-B*57:01 + DR7 + DQ3 – DRESS/AHSS	13	0	5	167	<0.01	72/100%	8%*	100/96.9%	∼12.5*	Mallal et al., 2002
5	Flupirtin – complex genotype HLA-DRB1*16:01 + DQB1*05:02 – DILI	11	0	614	10588	<0.01	1.8/100%	13.9:100,000*	100/94.5%	∼8000*	Nicoletti et al., 2016
6	Flucloxacilline – HLA-*57:01 – DILI	43	8	4	60	<0.01	84.3/93.75%	8.5:100,000	0.12/99.99%	∼13,000	Daly et al., 2009

*AHSS, abacavir hypersensitivity syndrome; Notation of 2x2 table in adaption of (Böhm et al., 2016): BE: Biomarker + Event, Be: Biomarker but no event, bE: event but no biomarker, be: neither biomarker nor event. Values marked with * were either extracted from additional source or calculated http://openvigil.pharmacology.uni-kiel.de/contingency-table-calculator.php. FD# HLADR denotes the number of dataset obtained from http://pgx.fudan.edu.cn/hladr/.*

(known) cohorts to the existing data (Nicoletti et al., 2016). The theoretical gold standard is to keep gathering reports until all cells can be filled in with sufficiently large counts which is often not feasible.

The full dataset (#4) using complex genotypes illustrates that the combination of genotypes enhances the performance of the test, in this case the PPV (Mallal et al., 2002).

The dataset #5 illustrates that even test with an extremely low sensitivity can lead to acceptable high PPV and NPV and thus NNS (Nicoletti et al., 2016).

The findings in dataset #6 shows a scenario in which testing appears very reasonable if calculating PPV and NPV from the original data (PPV = 91.49%, NPV = 88.23%, NNS = 1.3). However, when applying the real population-wide prevalence instead of relying solely on the counts reported in this publication, a much lower PPD of 0.12% results and thus a very high NNS of approximately 13,000 (Daly et al., 2009). Still, testing is desirable from the patient's point of view (NPV 100%). However, it is economically not feasible.

WHEN AND HOW TO TEST FOR A RISK OF HYPERSENSITIVITY

From a clinical point of view, testing for pharmacogenetics markers for the prediction of drug hypersensitivity reactions is only useful if

(i) a test with sufficient PPV/NPV exists,
(ii) an alternative treatment or diagnostic option exist that can be employed on a positive test result and
(iii) the test is unlikely to inflict damage on the patient.

From an economically point of view, it might be added:
(iv) testing of the population at risk should be cheaper than the costs to treat ADR in this population, implying a low NNS.

Of note, the NNS requires knowledge of the prevalence to be calculated. Due to the rareness of the events being analyzed, in most cases just estimates of the incidence-rates in a subpopulation (primarily users of the drug) are known. Consequently, NNS figures will vary depending on these epidemiologic data. **Table 4** lists the drugs for which currently a genetic testing prior to exposition is mandatory.

Sometimes, more than one test for a risk-predicting factor is available. Under most circumstances, phenotyping requires more time and could potentially damage the patient. E.g., the risk of a drug hypersensitivity reactions to abacavir can be assessed in a variety of ways: a genetic test for HLA-B*57:01, an *ex vivo* test like lymphocyte transformation test (LTT) and *in vivo* tests like a skin patch test or an oral challenge (Rive et al., 2013). The genetic test and the LTT bear no risk for the patient. The genetic test needs no cultivation of cells and is thus much cheaper than the LTT. The skin patch might induce a reaction and the oral challenge option cannot be used due to severe drug hypersensitivity reactions.

TABLE 4 | Drugs for which currently pharmacogenetics testing is mandatory.

Drug	Biomarker
Abacavir	HLA- B*57:01
Carbamazepine	Only for Thai and Han Chinese: HLA-B*15:02

Not included are any test for the existence or subsceptibility of the pharmacodynamic target (e.g., kinase inhibiting oncologic drugs and the required prior genetic testing for a mutation in the kinase).

Human leukocyte antigen typing can also be done using serology, i.e., antibodies directed at certain surface proteins. However, the accuracy of this method is lower (7.1% misassignments) than genetic HLA typing (Bozon et al., 1996). Subtle changes of the HLA-encoded proteins are usually not detectable with serology-based methods. It is doubtful whether binding pockets to drugs or other interacting proteins (e.g., T cell receptors) are being recognized by the currently employed antibodies in serology-based HLA typing.

Summarizing, genetic tests have been proven to be usually the safest, fastest, and cheapest screening tool.

CONCLUSION

Adverse drug reaction are a major burden for the health care system. A large percentage could be prevented. Pharmacogenetic testing can contribute to avoidance of ADR, both pharmacological ADR (type A) and drug hypersensitivity reactions (type B ADR). Certain alleles and complex genotypes of HLA genes contribute to drug hypersensitivity reactions. Common drug hypersensitivity reactions include cytotoxicity in skin, liver, and blood cells. The decision for HLA genotyping before drug therapy is dependent on severity of the expected ADR and the existence of other treatment options, as well as a reasonable high positive and NPVs of the test in question in the population to be analyzed.

AUTHOR CONTRIBUTIONS

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*HLA-B*58:01* for Allopurinol-Induced Cutaneous Adverse Drug Reactions: Implication for Clinical Interpretation in Thailand

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Background: The aim of this study was to investigate the predisposition to different types of allopurinol-induced cutaneous adverse drug reactions (CADR), including Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN; SJS-TEN, $n = 13$), drug reaction with eosinophilia and systemic symptoms (DRESS, $n = 10$) and Maculopapular eruption (MPE; $n = 7$), conferred by *HLA-B*58:01* in a Thai population.

Methods: This case-control association study compares 30 patients with allopurinol-induced CADR, allopurinol-tolerant control patients ($n = 100$), and a Thai general population ($n = 1095$). Patients' *human leukocyte antigen type B (HLA-B)* alleles were genotyped by using a two-stage sequence-specific oligonucleotide probe system.

Results: Of a total 30 patients with CADR due to allopurinol, 29 (96.7%) patients were found to be at least heterozygous for *HLA-B*58:01,* compared to only 4.0% in allopurinol-tolerant patients ($p < 0.001$). Odds ratio (OR) for the association of *HLA-B*58:01* with allopurinol-induced CADR in this population was 696.0 (95% CI: 74.8–6475.0). The *HLA-B*58:01* allele was present in all patients with allopurinol-induced SJS-TEN (OR = 579.0, 95%CI: 29.5–11362.7, $p < 0.001$) and DRESS (OR 430.3, 95%CI: 22.6–8958.9, $p < 0.001$). Additionally, OR of *HLA-B*58:01* was highly significant in the allopurinol-induced MPE patients (OR 144.0, 95%CI: 13.9–1497.0, $p < 0.001$).

Conclusion: In this study we confirmed the association between *HLAB*58:01* and allopurinol-induced SJS-TEN in a Thai population. In addition, we identified an association between *HLA-B*58:01* and allopurinol-induced DRESS and MPE in this population. Therefore, *HLA-B*58:01* can be used as a pharmacogenetic marker for allopurinol-induced CADR including SJS-TEN, DRESS and MPE. These results suggest that screening for *HLA-B*58:01* alleles in patients who will be treated with allopurinol would be clinically helpful in preventing the risk of developing CARD in a Thai patients.

Summary

- Regardless of phenotype, this is the first pharmacogenetic study of allopurinol-induced CADR in patients of Thai ancestry.
- In this study we confirmed the association between *HLA-B*58:01* and allopurinol-induced SJS-TEN, DRESS, and MPE in Thai population.
- Regarding to our findings, the pharmacogenetic interpretation could be generalized to drug hypersensitivity including DRESS, SJS-TEN, and MPE.

Keywords: *HLA-B*58:01*, allopurinol, Thai, SJS, TEN, DRESS, MPE, drug hypersensitivity

INTRODUCTION

In this last decade, pharmacogenetic studies have shown a strong association between *human leukocyte antigen (HLA)* alleles and susceptibility to drug hypersensitivity reactions (Sukasem et al., 2014). *HLA* genes are a major contributor to drug hypersensitivity involving direct stimulation of immune effector cells and imitating an allergic reaction (Adam et al., 2011; Cheng and Su, 2014). Currently, *HLA-B* alleles have been used as pharmacogenetic markers to predict drug-induced cutaneous adverse drug reactions (CADR; Daly, 2014; Sukasem et al., 2014). The CADR such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), drug reaction with eosinophilia and systemic symptoms (DRESS) or drug hypersensitivity syndrome (DHS), and acute generalized exanthematous pustulosis (AGEP) are often life-threatening. Maculopapular eruption (MPE) is recognized as a mild form of hypersensitivity reaction (Lonjou et al., 2008; Karlin and Phillips, 2014; Sasidharanpillai et al., 2015).

Clinical presentation of SJS and TEN is characterized by rapid progression of mucosal detachment and systemic symptoms which may present as fever, mild elevation of hepatic enzymes, and intestinal and pulmonary manifestation (Barvaliya et al., 2011; Harr and French, 2012). SJS and TEN are differentiated by the seriousness of skin detachment, which is limited in SJS (<10% of body surface area; BSA) and more widespread in TEN (>30% of BSA), with the intermediate stage (10–30% of BSA) of skin detachment referred to as SJS/TEN overlapping (Aihara, 2011; Harr and French, 2012). DRESS syndrome is an extremely serious adverse effect referred to sometimes as DHS. It is characterized by a skin rash, lymphadenopathy, fever, and can involve single or multiple organs (Aihara, 2011; Fleming and Marik, 2011).

Allopurinol is a commonly prescribed medication that has been used to inhibit xanthine oxidase in patients with gouty arthritis, hyperuricemia, and in cancer patients undergoing chemotherapy (Lam et al., 2013; Min et al., 2015). In Thailand, allopurinol is a major cause of CADR and has been reported as the second most frequent cause of CADR, including SJS-TEN (SJS, TEN, SJS/TEN) and DRESS (Tassaneeyakul et al., 2009; Saokaew et al., 2014). Several studies have reported that severe reactions to allopurinol are strongly associated with *HLA-B*58:01*, which is carried by 8–15% of Han Chinese and Thais (Hung et al., 2005; Tassaneeyakul et al., 2009; Puangpetch et al., 2015), but occurs relatively infrequently in Japanese (0.6%) and European (0.8%) populations (Kaniwa et al., 2008; Lonjou et al., 2008; Goncalo et al., 2013). According to the data from the spontaneous reports by the Health Product Vigilance Center of Thailand, allopurinol is the second ranked of common culprit drugs, with at least 1488 patients suffering from SJS-TEN and at least 75 patients suffering from DRESS during the last 20 years (http://thaihpvc.fda.moph.go.th/thaihvc/Public/News/uploads/hpvc_5_13_0_100526.pdf).

A previous publication from Thailand showed that the *HLA-B*58:01* allele is a strong marker for allopurinol-induced CADR in the Thai population (Tassaneeyakul et al., 2009). However, that study reported only an association between allopurinol-induced SJS-TEN and *HLA-B*58:01*. More recently, a high frequency of *HLA-B*58:01* was reported in Portuguese patients with allopurinol-induced DRESS also (Goncalo et al., 2013). Therefore, this study aims to determine the association of allopurinol-induced CADR, which includes DRESS and SJS-TEN, and also MPE, with the *HLA-B*58:01* allele in Thai patients.

MATERIALS AND METHODS

Subjects and Characteristics

In this study, we carried out research as a retrospective and prospective case-control study. From 2011 to 2015, patients with allopurinol-induced CADR admitted to the allergy clinic of Faculty of Medicine Ramathibodi Hospital, Mahidol University were enrolled. Thirty patients with allopurinol-induced CADR were categorized into DRESS (10 cases), SJS-TEN (13 cases) and MPE (7 cases). Patients who had been taking allopurinol for more than 6 months without evidence of cutaneous adverse effects were recruited as allopurinol-tolerant controls ($n = 100$). In addition, general population who had not taken allopurinol and had no history of drug induced cutaneous adverse reactions were included in this study. Both case and control subjects were independently recruited with no family relationship. Data for this healthy control group was obtained from 1095 subjects undergoing *HLA-B* genotyping through the Laboratory for Pharmacogenomics, Somdech Phra Debaratana Medical Center (SDMC), Ramathibodi Hospital, Thailand.

Abbreviations: AGEP, Acute generalized exanthematous pustulosis; BSA, Body surface area; CADR, Cutaneous adverse drug reactions; DRESS, Drug reaction with eosinophilia and systemic symptoms; DHS, Drug hypersensitivity syndrome; HLA, Human leukocyte antigen; MPE, Maculopapular eruption; SJS, Stevens-Johnson syndrome; TEN, Toxic epidermal necrolysis; PPV, Positive predictive value; NPV, Negative predictive value.

The study was performed and approved by the Ramathibodi Hospital ethical review board, and informed consent was obtained from all of the participants.

Diagnosis of Cutaneous Adverse Drug Reactions (CADR)

All CADR patients were assessed by a dermatologist and allergist who reviewed photographs, pathological slides, clinical morphology, and medical records. The diagnosis of drug-induced DRESS, SJS-TEN was made according to the RegiSCAR criteria (Choudhary et al., 2013). In brief, DRESS was diagnosed in patients presenting with fever, maculopapular rash with internal organ involvement, and hematologic abnormalities. SJS was diagnosed in patients with skin rash and mucosal erosion covering up to 10% of BSA whereas SJS-TEN overlap was diagnosed in patients with epidermal necrosis whose blistering skin lesions affected between 10 and 30% of BSA. MPE was diagnosed in patients presenting with danger signs in drug-induced exanthema or covering 30% BSA with or without associated systemic symptoms, but not fulfilling criteria for DRESS (Pichler et al., 2002).

Genomic DNA Extraction

Blood samples were collected into EDTA tubes. DNA was isolated using the MagNA Pure automated extraction system (Roche Diagnostics, USA) based on magnetic-bead technology. The quality of genomic DNA was assessed using a Nano Drop ND-1000 to measure quantity and purity of genomic DNA. All DNA was aliquotted and stored at −20°C before analysis.

HLA-B Typing

The *HLA-B* alleles were genotyped by the Polymerase Chain Reaction-sequence specific oligonucleotide probe (PCR-SSOP) principles with the commercial kit (LABType SSO HLA Typing Kit; One Lambda Inc., CA, USA). Then, the *HLA-B* alleles were carried out using Luminex™ Multiplex Technology (Luminex® IS 100, USA). Briefly, PCR products were hybridized against a panel of oligonucleotide probes coated on polystyrene microspheres that have sequences complementary to stretches of polymorphic sequence within the target *HLA-B* alleles. The amplicon-probe complex was visualized using a colorimetric reaction and fluorescence detection technology. Data analysis for the *HLA-B* assays were performed with HLA fusion™ 2.0 software.

Statistical Analysis

The association between *HLA-B*58:01* and allopurinol-induced CADR was evaluated by comparing the group of individuals with CADR with the allopurinol-tolerant groups and the general population. Data were counted by presence or absence of *HLA-B*58:01* allele. Chi-square test and Fisher's exact test were used to analyze the association between allopurinol-induced cutaneous adverse reactions and *HLA-B*58:01*. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The strength of association was estimated by calculating the odds ratio (OR) with a 95% confidence interval (CI). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. $P \leq 0.05$ (two-sided) were considered to indicate statistical significance.

RESULTS

Subjects Clinical Characteristics

Of the 30 patients with CADR, 23 had underlying gout and seven had hyperuricemia. Seventeen (56.7%) patients were male and 13 (43.3%) were female, with an average age of 73.3 years (range 30–88 years). The mean duration of allopurinol use was 16.4 ± 14.3 days with a mean dosage of 239.29 ± 87.0 mg/day (range, 100–600 mg/day).The mean interval from allopurinol initiation to symptom onset was 22.2 ± 12.9 days (range, 7–42 days). The onset of symptoms for all patients was within the first 2 months of allopurinol exposure. The most common underlying were Hypertension consisting of 20 patients (66.7%) followed by Chronic Kidney Disease (CKD) with 13 patients (43.3%) and Diabetes with 4 patients (13.3%). The most common of co-medication was colchicine (19/30; 63.3%). Patient's characteristics, duration of allopurinol exposure to symptom onset and the results of *HLA-B* genotyping are summarized in **Tables 1, 2**.

TABLE 1 | Association of demographic data and Allopurinol-induced cutaneous adverse drug reactions (CADR).

Demographic data	Case (*n* = 30)
SEX (n/%)	
Male	17/56.7
Female	13/43.3
Age (median/range)	73/30–88
UNDERLYING DISEASE (n/%)	
Hypertension	20/66.7
Chronic kidney disease	13/43.3
Diabetes	4/13.3
CO-MEDICATION	
Colchicine	19/63.3
Simvastatin	3/10.0
Prednisolone	2/6.7
DOSAGE OF ALLOPURINOL; mg/day (AVERAGE ± SD)	**239.29 ± 87.0**
DURATION OF ALLOPURINOL EXPOSURE; DAYS (AVERAGE ± SD)	**16.4 ± 14.3**
LABORATORY RESULT (AVERAGE ± SD)	
Uric acid; mg/dL	6.58 ± 2.537
Blood Urea Nitrogen; mg/dL	43.64 ± 32.341
Creatinine; mg/dL	2.45 ± 5.246
Aspartate aminotransferase; mg/dL	63.19 ± 88.120
Alanine transaminase; mg/dL	71.26 ± 96.188
PHENOTYPE (n/%)	
SJS-TEN	13/43.4
DRESS	10/33.3
MPE	7/23.3

*HLA-B*58:01* in Cutaneous Adverse Drug Reactions (CADR) Case-Control Study

To identify genetic markers for allopurinol–induced CADR including DRESS, SJS-TEN, and MPE, we carried out a case-control association study. Frequencies of *HLA-B*58:01* genotype in the three groups are shown in **Table 2**. Of the 30 patients with allopurinol-induced CADR, 29 patients (96.70%) carried *HLA-B*58:01*, while 4 of 100 (4.0%) allopurinol-tolerant controls and 111 of 1095 (10.1%) untreated controls carried this allele. The frequency of *HLA-B*58:01* in subjects with allopurinol-induced CADR was notably higher than in the allopurinol-tolerant group (OR 696.00; 95% CI: 74.81–6475.01, $p < 0.001$) and general population group (OR 257.08; 95% CI: 34.68–1905.57). In our *HLA-B* genotyping studies, no other alleles showed significant association with allopurinol-induced CADR (**Table 3**).

By extending our investigation for other HLA-B alleles, we found that *HLA-B*40:01* (29.0%, OR = 0.45; 95% CI, 0.09–2.13), *HLA-B*46:01* (25.0%, OR = 0.21; 95% CI, 0.05–0.96), and *HLA-B*51:01* (12.0%, OR = 0.25; 95%CI, 0.03–2.03) was detected more frequently in control group than in allopurinol-induced CADR groups (16.7, 6.7, and 3.3%, respectively). However, there were no statistically significant differences ($p > 0.05$) between case and control groups (**Table 3**).

TABLE 2 | Summary of characteristic and genotyping data of allopurinol-induced cutaneous adverse drug reactions (CADR) in individuals.

No.	Age/Sex	Phenotype	*HLA-B*58:01*	*HLA-B* genotyping
1	56/M	DRESS	Positive	*4402:5801*
2	72/F	SJS	Positive	*5701:5801*
3	78/F	SJS	Positive	*3501:5801*
4	68/F	SJS	Positive	*4601:5801*
5	85/F	MPE	Positive	*4001:5801*
6	48/F	SJS	Positive	*1301:5801*
7	68/M	SJS	Positive	*1301:5801*
8	74/M	DRESS	Positive	*5201:5801*
9	54/M	SJS-TEN	Positive	*4001:5801*
10	78/M	SJS-TEN	Positive	*4403:5801*
11	74/F	SJS-TEN	Positive	*1502:5801*
12	28/M	DRESS	Positive	*3915:5801*
13	37/F	DRESS	Positive	*0801:5801*
14	67/M	MPE	Negative	*1301:5401*
15	81/F	DRESS	Positive	*1301:5801*
16	76/F	SJS	Positive	*5801:5801*
17	76/M	SJS	Positive	*4001:5801*
18	73/M	DRESS	Positive	*5201:5801*
19	76/F	SJS-TEN	Positive	*1513:5801*
20	73/F	SJS-TEN	Positive	*4601:5801*
21	55/M	SJS-TEN	Positive	*1502:5801*
22	55/F	DRESS	Positive	*5801:5801*
23	78/M	DRESS	Positive	*1502:5801*
24	81/M	DRESS	Positive	*5801:5801*
25	61/M	DRESS	Positive	*5101:5801*
26	88/F	MPE	Positive	*4001:5801*
27	79/M	MPE	Positive	*4001:5801*
28	73/M	MPE	Positive	*1301:5801*
29	72/M	MPE	Positive	*1802:5801*
30	84/M	MPE	Positive	*3901:5801*

*HLA-B*58:01* in SJS-TEN and DRESS Cases-Controls Study

The relationship between *HLA-B*58:01* and allopurinol-induced SJS-TEN and DRESS was subsequently studied in this study. All 23 (100%) patients with allopurinol-induced SJS-TEN ($n = 13$) and DRESS ($n = 10$) cases had *HLA-B*58:01* (three patient was homozygous for *HLA-B*58:01*). As shown in **Table 4**, the *HLA-B 58:01* allele occurred at significantly increased frequencies among the allopurinol-induced SJS-TEN patients compared to the two control groups (OR = 579.00, 95%CI: 29.50–11362.67 and OR = 238.40, 95%: 14.08–4037.80). Sensitivity and specificity of *HLA-B*58:01* for prediction of allopurinol-induced SJS-TEN were 100.00 and 96.0%. In addition, the PPV and NPV of the *HLA-B*58:01* allele was also 76.47 and 100.0%, respectively (**Table 7**).

In addition, the *HLA-B*58:01* allele was associated with a higher risk of DRESS (OR 430.33, 95%CI: 22.64–8958.88, $p < 0.001$ and OR 185.42, 95%CI: 10.79–3185.84, $p < 0.001$) when compared with allopurinol tolerant patients and the general population, respectively (**Table 5**). Sensitivity and specificity of *HLA-B*58:01* for prediction of allopurinol-induced DRESS were 100.00 and 96.0%. In addition, the PPV and NPV of

TABLE 3 | The association of individual *HLA-B* allele with allopurinol-induced cutaneous adverse drug reactions (CADR).

HLA-B allele	Allopurinol-induced CADR ($n = 30$) (%)	Allopurinol tolerant control ($n = 100$) (%)	General population ($n = 1095$) (%)	CADR case vs. allopurinol tolerant control		CADR case vs. general population	
				OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
58:01	**29 (96.7)**	**4 (4.0)**	**111 (10.1)**	**696.00 (74.81–6475.01)**	**<0.001**	**257.08 (34.68–1905.57)**	**<0.001**
13:01	5 (16.7)	9 (9.0)	137 (12.5)	2.02 (0.62–6.58)	0.242	1.40 (0.53–3.71)	0.501
15:02	3 (10.0)	20 (20.0)	161 (14.7)	0.44 (0.12–1.6)	0.218	0.65 (0.19–2.15)	0.475
40:01	5 (16.7)	29 (29.0)	162 (14.8)	0.49 (0.17–1.40)	0.184	1.15 (0.43–3.05)	0.776
46:01	2 (6.7)	25 (25.0)	227 (20.7)	0.21 (0.05–0.96)	0.051	0.27 (0.06–1.16)	0.077
51:01	1 (3.3)	12 (12.0)	65 (5.9)	0.25 (0.03–2.03)	0.196	0.55 (0.07–4.08)	0.556

the *HLA-B*58:01* allele was also 76.43 and 100.0%, respectively (**Table 7**).

*HLA-B*58:01* in MPE Cases-Controls Study

Of the seven patients with allopurinol-induced MPE, 6 of 7 (85.7%) patients had *HLA-B*58:01* whereas 4 (4.0%) of allopurinol-tolerant patients had *HLA-B*58:01*. The one patient with MPE who did not have the *HLA-B*58:01* allele carried *HLA-B*13:01/54:01*. In this study, the OR of *HLA-B*58:01* was highly significant in the allopurinol-induced MPE patients (OR 144.00, 95%CI: 13.85–1497.03, $p < 0.001$), as shown in **Table 6**. Sensitivity and specificity of *HLA-B*58:01* for prediction of allopurinol-induced MPE were 85.71 and 96.0%, respectively. In addition, the PPV and NPV of the *HLA-B*58:01* allele was also 60.0 and 98.97%, respectively (**Table 7**).

DISCUSSION

In the present study, the case–control analysis included 30 cases of allopurinol-induced CADR, which included DRESS (10 cases), SJS-TEN (13 cases), and MPE (7 cases). The association study in Thai patients examined only a limited phenotype of allopurinol-induced SJS-TEN (Tassaneeyakul et al., 2009). In this study we confirmed the association between *HLA-B*58:01* and allopurinol-induced SJS-TEN (OR = 579.0) in a Thai population. In addition, we identified an association between *HLA-B*58:01* and allopurinol-induced DRESS and MPE with OR 430.3 and 144.0, respectively. Thus, the *HLA-B*58:01* is associated with allopurinol-induced CADR including SJS-TEN, DRESS and MPE in a Thai population.

*HLA-B*58:01* was the most predominant allele associated with allopurinol-induced CADR and was not found in only one patient who had allopurinol-induced MPE (*HLA-B*13:01/54:01*).

TABLE 4 | The association of individual *HLA-B* allele with allopurinol-induced SJS-TEN.

HLA-B allele	Allopurinol-induced SJS-TEN ($n = 13$) (%)	Allopurinol tolerant control ($n = 100$) (%)	General population ($n = 1095$) (%)	SJS-TEN cases vs. Allopurinol tolerant control		SJS-TEN cases vs. general population	
				OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
58:01	**13 (100.0)**	**4 (4.0)**	**111 (10.1)**	**579.00 (29.50–11362.67)**	**<0.001**	**238.40 (14.08–4037.80)**	**<0.001**
13:01	2 (15.4)	9 (9.0)	137 (12.5)	1.84 (0.35–9.62)	0.471	1.27 (0.28–5.80)	0.756
15:02	2 (15.4)	20 (20.0)	161 (14.7)	0.73 (0.15–3.55)	0.694	1.05 (0.23–4.80)	0.945
40:01	2 (15.4)	29 (29.0)	162 (14.8)	0.45 (0.09–2.13)	0.312	1.05 (0.23–4.77)	0.953
46:01	2 (15.4)	25 (25.0)	227 (20.7)	0.55 (0.11–2.63)	0.450	0.70 (0.15–3.16)	0.638
51:01	0 (0.0)	12 (12.0)	65 (5.9)	0.26 (0.01–4.69)	0.363	0.58 (0.03–9.91)	0.709

TABLE 5 | The association of individual *HLA-B* allele with allopurinol-induced DRESS.

HLA-B allele	Allopurinol-induced DRESS ($n = 10$) (%)	Allopurinol tolerant control ($n = 100$) (%)	General population ($n = 1095$) (%)	DRESS cases vs. allopurinol tolerant control		DRESS cases vs. general population	
				OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
58:01	**10 (100.0)**	**4 (4.0)**	**111 (10.1)**	**430.33 (22.64–8958.88)**	**<0.001**	**185.42 (10.79–3185.84)**	**<0.001**
13:01	1 (10.0)	9 (9.0)	137 (12.5)	1.12 (0.13–9.90)	0.917	0.78 (0.10–6.18)	0.812
15:02	1 (10.0)	20 (20.0)	161 (14.7)	0.44 (0.05–3.72)	0.454	0.64 (0.08–5.12)	0.678
40:01	1 (10.0)	29 (29.0)	162 (14.8)	0.27 (0.03–2.25)	0.225	0.64 (0.08–5.08)	0.673
46:01	0 (0.00)	25 (25.0)	227 (20.7)	0.14 (0.01–2.49)	0.181	0.18 (0.01–3.11)	0.240
51:01	1 (10.0)	12 (12.0)	65 (5.9)	0.82 (0.09–7.01)	0.852	1.76 (0.22–14.11)	0.594

TABLE 6 | The association of individual *HLA-B* allele with allopurinol-induced MPE.

HLA-B allele	Allopurinol-induced MPE ($n = 7$) (%)	Allopurinol tolerant control ($n = 100$) (%)	General population ($n = 1095$) (%)	MPE case vs. Allopurinol tolerant control		MPE case vs. general population	
				OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
58:01	**6 (85.7)**	**4 (4.0)**	**111 (10.1)**	**144.00 (13.85–1497.03)**	**<0.001**	**53.19 (6.35–445.85)**	**<0.001**
13:01	2 (28.6)	9 (9.0)	137 (12.5)	4.04 (0.68–23.91)	0.123	2.80 (0.54–14.56)	0.222
15:02	0 (0.0)	20 (20.0)	161 (14.7)	0.26 (0.01–4.77)	0.366	0.39 (0.02–6.79)	0.515
40:01	3 (42.9)	29 (29.0)	162 (14.8)	1.84 (0.39–8.72)	0.445	4.32 (0.96–19.48)	0.057
46:01	0 (0.0)	25 (25.0)	227 (20.7)	0.20 (0.01–3.58)	0.272	0.25 (0.01–4.47)	0.349
51:01	0 (0.0)	12 (12.0)	65 (5.9)	0.47 (0.03–8.78)	0.615	1.05 (0.06–18.57)	0.974

TABLE 7 | Sensitivity/Specificity/Positive predictive value (PPV)/Negative predictive value (NPV).

*HLA-B*58:01*	vs. allopurinol tolerant control (%)				vs. general population (%)			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
CADR	96.67	96.00	87.88	98.97	96.67	89.86	20.71	99.90
SJS-TEN	100.00	96.00	76.47	100.00	100.00	89.86	10.48	100.00
DRESS	100.00	96.00	76.43	100.00	100.00	89.86	8.26	100.00
MPE	85.71	96.00	60.00	98.97	85.71	89.86	5.13	99.90

Moreover, there was no significantly associated CADR with any other *HLA-B* alleles such as *HLA-B*13:01*, indicating that *HLA-B*58:01* has an important role in the progression of allopurinol-related CADR in the Thai population. The odds ratio (OR) for the association of *HLA-B*58:01* with combined CADR phenotypes in this population was 696.00 ($p < 0.01$). Using the allopurinol tolerant group as the control, the *HLA-B*58:01* allele had 95.20% sensitivity and 100% specificity for diagnosing CADR. This strong association also has been observed in other Asian countries (Hung et al., 2005; Kaniwa et al., 2008; Jung et al., 2011; Kang et al., 2011).

Previous study has proposed association of immune mechanisms in the development of several forms of allopurinol-induced CADR. Hung et al. has shown that the *HLA-B*58:01* allele is a strong genetic factor in the incidence of CADR (SJS-TEN, and DRESS) for Han Chinese taking allopurinol (Hung et al., 2005). Although, Tassaneeyakul et al. was the first to identify an association between *HLA-B*58:01* and allopurinol-induced SJS-TEN in Thai (Tassaneeyakul et al., 2009), no published data have yet confirmed such a strong correlation of *HLA-B*58:01* and allopurinol-induced DRESS and MPE in Thai patients. Recently, we identified an association between *HLA-B*58:01* and allopurinol-induced DRESS and MPE in Thai population.

This finding reveals that the risk of developing DRESS among those allopurinol users with *HLA-B*58:01* is significantly increased by 430.3 times compared to allopurinol-tolerant controls. The association is 100% in that the *HLA-B*58:01* was present in all 10 patients with allopurinol-induced DRESS, similar to the study in Han Chinese and Japanese populations (Hung et al., 2005; Kaniwa et al., 2008). In this study, we also confirmed a strong association between the allele *HLA-B*58:01* and susceptibility to allopurinol-induced SJS-TEN in Thai patients. Based on the strong association of the presence of *HLA-B*58:01* and DRESS and SJS-TEN, it is presumed that the attributable risk of CADR due to the existence of this allele is larger—as high as 8% in Thailand (Puangpetch et al., 2015)—indicating that *HLA-B*58:01* is associated with the pathogenesis of allopurinol-induced CADR regardless of the phenotype or severity. This is in contrast to carbamazepine-induced SJS-TEN, with which *HLA-B*15:02* only shows association with only SJS-TEN (Suresh Kumar et al., 2005; Phillips et al., 2011; Lee et al., 2014).

Moreover, MPE has been considered to be distinct from SJS-TEN, characterized as macule and papule rash, with symmetry on both left and right of body and especially on the face, palms and feet, and with no detachment on the body surface area and no systemic involvement (Pichler et al., 2002). The association of *HLA-B*58:01* with MPE is less well studied (Profaizer and Eckels, 2012). Recently, Cao et al. found that all 22 Han Chinese with MPE in that study were *HLA-B*58:01* positive (Cao et al., 2012). Among 12 Australian patients, none was *HLA-B*58:01* positive, and three of four had MPE, which was statistically significantly different from the allopurinol-tolerant group (Lee et al., 2012). In recent times, we investigated an association between *HLA-B*58:01* and allopurinol-induced MPE. By comparison, the OR of *HLA-B*58:01* was 144.0 and 53.2 between the MPE patients with the allopurinol-tolerant and untreated Thai population groups. However, a major limitation of this study was the sample size of the MPE patients available for the analysis. Confirmation of this result in an independent cohort of larger sample size would allow us to determine whether the *HLA-B*58:01* identified in this study is definitely associated with the development of allopurinol-induced MPE and provide more accurate estimates of their impact in the clinical practice.

Commonly, allopurinol is associated with CADR ranging from mild skin rash, such as MPE to life-threatening severe cutaneous adverse reactions including DRESS and SJS-TEN (Ng et al., 2016). Interestingly, *HLA-B*40:01* was found much more frequently in the allopurinol-induced MPE ($n = 3/7$; 42.9%) than others allopurinol-induced SJS-TEN ($n = 2/13$; 15.4%) and no *HLA-B*40:01* was observed in allopurinol-induced DRESS cases. Furthermore, homozygous *HLA-B*58:01* was found only in severe cutaneous adverse drug reaction, that included SJS-TEN and DRESS. Hence, the complementary alleles with *HLA-B*58:01* carriers might be identified as a marker influencing susceptibility to different types of allopurinol-induced CADR in Thai population. Moreover, Grover et al. found that *HLA-B*40:01* could be a protective marker for carbamazepine-induced CADR (OR = 0.32; 95% CI = 0.19–0.53; $P = 1.08 \times 10^{-5}$; Grover and Kukreti, 2013). In this study, *HLA-B*40:01* (29.0%), *HLA-B*46:01* (25.0%), and *HLA-B*51:01* (12.0%) was higher in allopurinol-tolerant than in allopurinol-induced CADR groups (16.7, 6.7, and 3.3%, respectively, $p > 0.05$). This possibly suggests that the both alleles might be protective markers for allopurinol-induced CADR. However, the number of patients may not be enough to reveal all the assumptions, further investigation using a large number of samples and well-designed study is required to better understand.

Practically, colchicine is an immune-modulating agent which is normally prescribed with allopurinol for acute gout

FIGURE 1 | Pharmacogenomics card and clinical interpretation; (A) clinical interpretation before this study, (B) revised clinical interpretation after this study.

prophylaxis. Ryu et al. found that the use of colchicine was the clinical risk factor for adverse events when using allopurinol (Ryu et al., 2013). In this study, 63.3% ($n = 19/30$) of patients with allopurinol-induced CADR were treated with colchicine as a co-medication. Both allopurinol and colchicine are potential offending drugs in the present case of CADR. Commonly, allopurinol, one of the most frequent causes of SJS and TEN. Colchicine is also associated, but to a lesser degree (Ryu et al., 2013). However, the risk of allopurinol-induced CADR in Concurrent administration with colchicine in patients carried *HLA-B*58:01* allele has not yet been evaluated.

With evidence support the association of the *HLA-B*58:01* allele with allopurinol-induced CADR, CPIC guidelines recommend the use of pharmacogenomics tests for presence of the *HLA-B*58:01* allele before initiating allopurinol therapy in patients (Hershfield et al., 2013). After patients have taken an HLA test, their results are entered into a plastic "pharmacogenomic wallet card," which basically contains the genomic results of those related to the risk of CADR. This card can be carried around and shown to different doctors in the future (**Figures 1A,B**). Currently, there were over 1400 patients which were genotyped and delivered the pharmacogenomic cards for screening *HLA-B*58:01* before allopurinol prescription from our setting. The pharmacogenetics interpretation has been changed from "High risk for allopurinol-induced SJS-TEN" (**Figure 1A**) to be "High risk for allopurinol-induced SJS-TEN, DRESS and MPE" (**Figure 1B**) from our finding in Thai population. Physicians and national policy makers should consider genetic screening for the *HLA-B*58:01* alleles prior to initiation of allopurinol therapy in Thai patients.

In summary, a strong association between allopurinol-induced CADR and the *HLA-B*58:01* allele was confirmed. Incidence of the *HLA-B*58:01* allele is strongly associated with individuals who are at risk for allopurinol-induced DRESS, SJS-TEN, and MPE in the Thai population. Our results suggest that the screening tests for the *HLA-B*58:01* allele in patients who will be treated with allopurinol would be clinically helpful in reducing the risk of developing CADR.

AUTHOR CONTRIBUTIONS

CS designed and operated project, set goals and controlled project, analyzed results, and supervised the pharmacogenetic section. TJ assisted to coordinate project between pharmacogenetic part and clinical part. PK collected samples and extracted genomic DNA. AP analyzed statistical data. NK performed HLA typing. PS collected samples and performed HLA typing. PS managed clinical part and counseled all patients. JK controlled project, evaluated the effectiveness of treatment and advised the methodology. TR co-designed and co-operated project, controlled the operations to meet the goal, managed clinical part, and counseled all patients.

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MINI REVIEW
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MinION: A Novel Tool for Predicting Drug Hypersensitivity?

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The launch of the MinION Access Program has caused much activity within the scientific community. MinION represents a keenly anticipated, novel addition to the current melange of commercial sequencers. Driven by the nanopore sequencing mechanism that requires minimal sample manipulation, the device is capable of generating long sequence reads in sizes (up to or exceeding 50 kb) that surpass those of all other platforms. One notable advantage of this feature is that long-range haplotypes can be more accurately resolved; such advantage is particularly pertinent to the genotyping of complex loci such as genes encoding the human leukocyte antigens, which are pivotal determinants of drug hypersensitivity. With this timely, albeit brief, review, we set out to examine the applications on which MinION has been tested thus far, the bioinformatics workflow tailored to the unique characteristics of its extended sequence reads, the device's potential utility in the detection of genetic markers for drug hypersensitivity, and how it may eventually evolve to become fit for diagnostic purposes in the clinical setting.

Keywords: MinION, nanopore sequencing, drug hypersensitivity, sequence-based HLA typing, bioinformatics analysis

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INTRODUCTION

The launch of the MinION Access Program by Oxford Nanopore Technologies (ONT), a UK-based company specializing in nanopore sequencing, has caused much activity within the scientific community. The device is a miniature, third-generation sequencer in which 512 nanopores are housed and responsible for sensing single-stranded DNAs. With steadily improving sequencing accuracy, MinION has been a much welcome addition to the melange of tools deployed for diagnosing inherited drug hypersensitivity. The compact measurements of the device confer it such a degree of portability that is unsurpassable by other platforms. There has even been speculation that MinION could be transported to Mars and used to probe the existence of alien life forms (Check Hayden, 2015). Thus far, the use of MinION has been directed largely toward DNA sequencing; however, a broader range of MinION applications for RNA, microRNA, and protein analysis are being explored. A scaled-up version of MinION, which is composed of 48 flow cells and designated PromethION, has also been made available via another program, granting the participants early access to the platform (Karow, 2015).

MinION: HISTORY AND APPLICATIONS

The characteristics of ion channels as nanopores for DNA molecule detection have been extensively investigated since two decades ago (Kasianowicz et al., 1996; Howorka et al., 2001). When purified,

single-stranded DNAs are passed through an array of pores embedded onto a membrane, characteristic current patterns that reflect the identities of DNA bases are produced (Clarke et al., 2009; Ip et al., 2015). Various improvements in the experimental setup have increased the accuracy of base-reading; these have included modification of the structure of α-hemolysin channels, addition of bulky cyclodextrin molecules to reduce the speed of DNAs translocating through the pores, introduction of hairpin polynucleotides to connect unzipped, double-stranded DNAs, and adjustment of salt concentrations in the buffer to alter the voltage across the membrane (Clarke et al., 2009; Brown et al., 2012). These changes have ultimately led to the release of MinION. Compared with existing second-generation sequencers such as MiSeq and Ion Torrent that sequence up to 400 bp, MinION is deemed ideal for DNA sequencing as it can generate long reads up to or exceeding 50 kb (Jain et al., 2015). Moreover, it does not require pre-amplification of the sample, which removes potential bias in the data that could be introduced by polymerase chain reactions (PCRs).

MinION has been tested in diverse applications, ranging from early work on bacterial sequencing and identification, to recent discovery of its ability to detect pathogens in human plasma and that to distinguish methylated DNA bases from their unmethylated counterparts (**Figure 1**). Extended sequences generated by MinION have been used to supplement better quality MiSeq data in constructing the genomes of *Bacteroides fragilis* and *Saccharomyces cerevisiae* (Goodwin et al., 2015; Risse et al., 2015). Further enhancement of bioinformatics workflow has allowed *de novo* genome assembly for the *Escherichia coli* K-12 MG1655 strain, whereby 98.4% nucleotide accuracy was noted across the 4.6-mb reconstructed genome (Loman et al., 2015). Such high-quality data output from the device has later been translated into differentiating three closely related poxviruses, namely cowpox, vaccinia-MVA, and vaccinia-Lister (Kilianski et al., 2015). The portability of MinION and relatively rapid sample preparation and data generation associated with the sequencer have positioned it to be a useful tool in the recent Ebola epidemic, wherein it was used on-site for monitoring the evolution of Ebola virus in a series of clinical samples. Similar principles of viral identification from human blood have also been applied to sequencing chikungunya and hepatitis C viruses (Greninger et al., 2015; Quick et al., 2016).

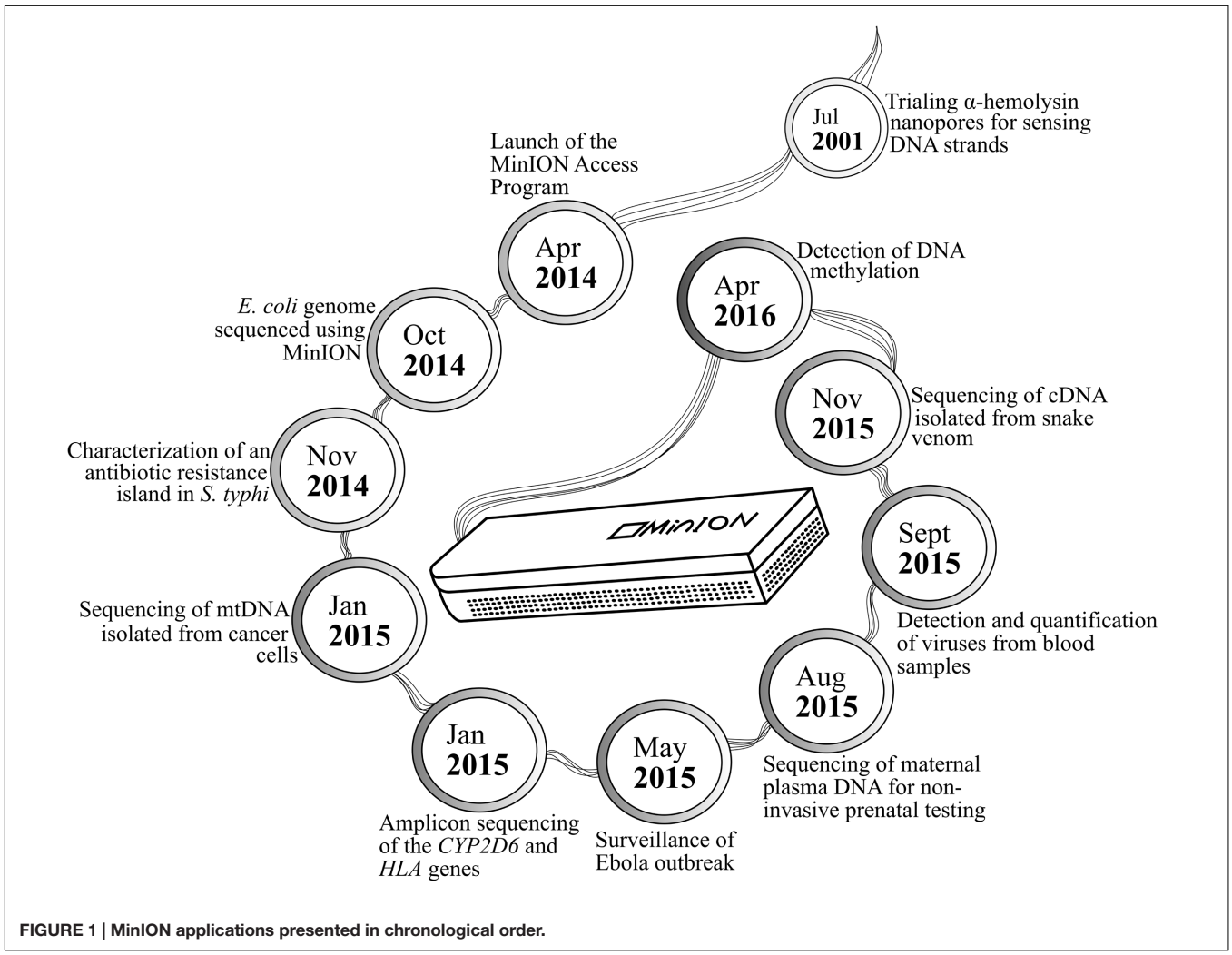

FIGURE 1 | MinION applications presented in chronological order.

In the detection of DNA methylation patterns in human samples, the subtle differences in the electrical signals produced by methylated cytosine bases could also be picked up by MinION (Simpson et al., 2016). This is an exciting breakthrough as the conventional identification of these molecular modifications requires a more complex and specialized sample preparation approach, such as methylated DNA immunoprecipitation followed by sequencing, or bisulfite genomic sequencing (Frommer et al., 1992; Corley et al., 2015). Bisulfite sequencing, the gold standard for detecting DNA methylation, indirectly allows differentiation of methylated cytosines via selective conversion of unmethylated cytosines to uracils, which are then detected as thymine *post*-PCR (Li and Tollefsbol, 2011). In contrast, the MinION-based workflow is much simpler: a statistical model has been developed and optimized for direct identification of 5-methylcytosine from samples prepared by ONT's standard protocol (Simpson et al., 2016). Though a direct causal relationship between drug hypersensitivity and DNA methylation has not been observed, the epigenetic phenomenon has been proven to mediate other processes such as disease pathogenesis and the effectiveness of a number of drugs (Beyrouthy et al., 2009; Anier et al., 2010).

PROCESSING OF NANOPORE SEQUENCING DATA

Central to the processing of nanopore sequencing data is the hidden Markov model (Eddy, 2004), which has been applied to various stages of sequence analysis from base-calling, fine-tuning alignment, to variant discovery (Jain et al., 2015; Szalay and Golovchenko, 2015). For base-calling, this model yields statistical deductions about the underlying DNA sequences (hidden state) based on a series of *emitted* observations. The successive ionic perturbations (events) caused by 6-mer DNAs (or 5-mer DNAs for now-obsolete workflows) that are drawn through the nanopores are rendered into DNA sequences based on known pairs of DNA sextets and corresponding current values (Timp et al., 2012; Szalay and Golovchenko, 2015). A major obstacle to this approach is that the current levels for all possible 6-mer combinations comprise a continuum of electrical signals rather than segregate into discrete patterns that could be unambiguously interpreted. To tease out these ionic signatures, additional clues are gained from neighboring sequences (Timp et al., 2012). For instance, suppose we were to infer a 6-mer DNA from an electrical signal, which indicated there were two possibilities: TACGTA and TACGTT. We knew that, on most occasions, the preceding sequence, ATACGT, was likely to transition to TACGTA; thus TACGTA should have been the sequence motif from which the signal had originated (Timp et al., 2012). Though this example is rather simplistic, it serves to illustrate the effectiveness of the Markov model. The emission and transition probabilities can be derived from datasets that are used to train the model.

Similarly, for sequence alignment, *maximum likelihood estimates* can be computed for all nanopore sequencing error types within a Markov network, i.e., insertions, deletions, and substitutions. These estimates are then used to ascertain whether a reference-discordant read is indeed misaligned. For instance, as A–T or T–A miscalls are unlikely, sequence alignments containing these mismatches may well have been incorrectly placed. Alternately, the aligner may not be at fault and the discrepancies may have arisen from inherent DNA variation (Jain et al., 2015). This strategy has been extrapolated to enhance alignments generated by existing aligners and subsequently detect variants deliberately introduced into a phage genome reference (Jain et al., 2015).

An example of a bioinformatics pipeline for MinION-generated data is shown in **Figure 2**. Raw electrical signals can be base-called by a tool provided by ONT, Metrichor, or other open-source software (Boža et al., 2016; David et al., 2016). DNA sequences produced from the nanopores are stored in the FAST5 format, alongside other types of data such as the run statistics. All data are stratified and placed within predefined categories. Pre-alignment processing typically requires extraction of FASTA or FASTQ sequences from the FAST5 files, using Poretools (Python-based; Loman and Quinlan, 2014) or poRe (written in R; Watson et al., 2015). A number of long-read aligners have been tested thus far on nanopore sequences, including BLASR, BWA-MEM, LASTZ, and LAST. One of the challenges unique to long-read alignment lies in quickly finding short matches, termed *seeds* or *anchors*, between two sequences during a preliminary round of alignment (Li and Durbin, 2009; Chaisson and Tesler, 2012; Li, 2013). With longer sequences, the numbers of possible matches and mismatches are larger; hence, the operation of the aligners would be affected, to a greater extent, by the efficiency of their *seed-and-extend* algorithm.

BLASR and BWA-MEM first invoke *Burrow-Wheelers transform* to create easily searchable, sorted strings (index) of a reference genome to facilitate initial side-by-side check that pinpoints short *exact* matches, which are subsequently extended or refined to form longer DNA stretches (Chaisson and Tesler, 2012; Li, 2013). LASTZ resembles BLASR and BWA-MEM but with two principal variations. First, the aligner employs a different indexing mechanism and dissects the reference sequence into equally sized, overlapping segments to ease sequence comparison. Second, it does not require perfect similarity for qualifying a short match as an acceptable seed; some degree of discrepancy is permitted (Harris, 2007). LAST differs from LASTZ in that it can resolve repeat-rich sequences more successfully (Kiełbasa et al., 2011). When aligned, the sequences can be further scrutinized using NanoOK, which works out the read-length distribution, the occurrence of k-mers, the depths of coverage across targets, and other relevant statistics (Leggett et al., 2015). With accurate alignments, high-confidence DNA variant calls can then be generated.

PHARMACOGENETICS: A CLASSIC CASE OF ABACAVIR HYPERSENSITIVITY

Abacavir, a nucleoside reverse-transcriptase inhibitor used to treat HIV-1 infection, causes a potentially fatal hypersensitivity reaction, to which Caucasians are notably susceptible, in 4–9%

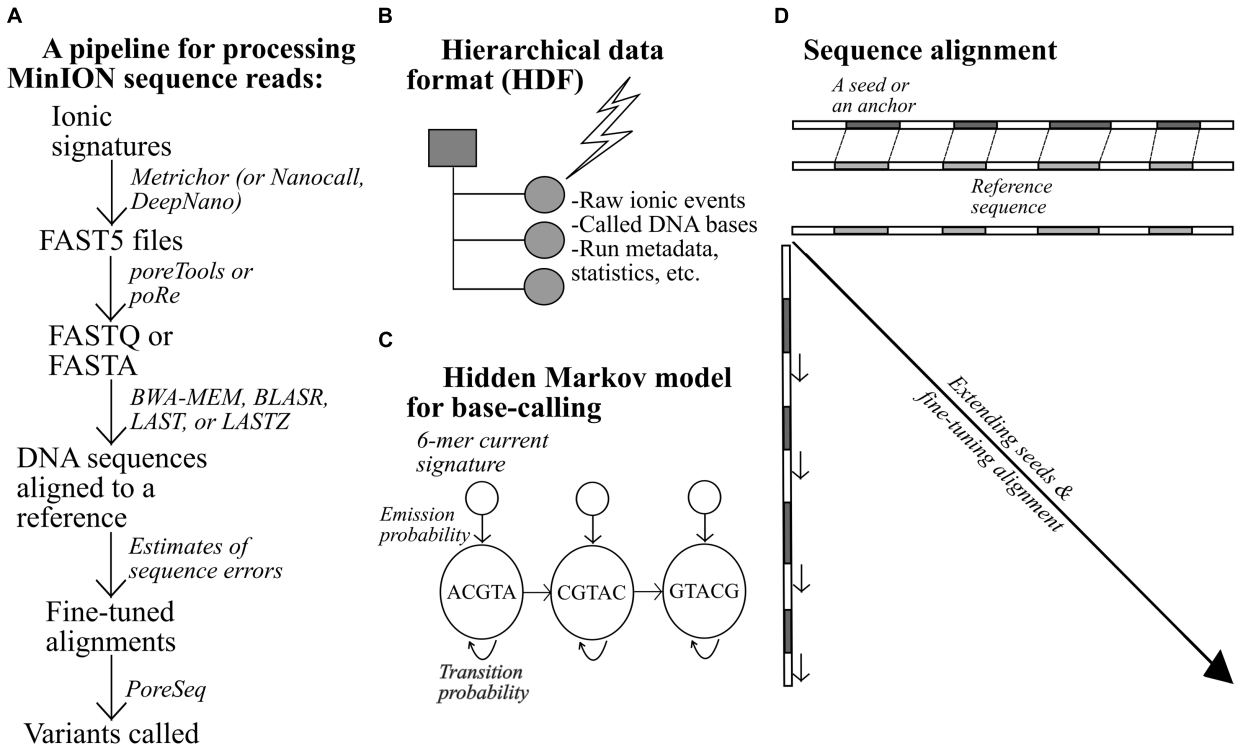

FIGURE 2 | An example of a pipeline for the processing of MinION data (A), (B–D) alongside additional notes for relevant bioinformatics-related concepts.

of individuals exposed to the drug (Hetherington et al., 2002; Mallal et al., 2002; Symonds et al., 2002; Martin et al., 2004). Abacavir-induced hypersensitivity remains, to date, one of the few rewarding examples of pharmacogenetics-guided therapy. Genetic predisposition, specifically the presence of the *HLA-B*57:01* allele (human leukocyte antigen), is a strong predictor of abacavir hypersensitivity. The association, first reported in a Western Australian population (Mallal et al., 2002), has since been replicated in many other studies (Hetherington et al., 2002; Hughes et al., 2004a; Rauch et al., 2006; Rodriguez-Novoa et al., 2007; Waters et al., 2007; Zucman et al., 2007; Mallal et al., 2008). The *HLA-B*57:01* allele has a high degree of penetrance; the pooled odds ratio from three studies was computed to be 29. Nevertheless, not all *HLA-B*57:01* carriers would be hypersensitive toward abacavir; of every ten individuals harboring the allele, only five would indeed develop a reaction (Hetherington et al., 2002; Mallal et al., 2002; Hughes et al., 2004a). It is likely that other gene loci or pathways, particularly those implicated in the conversion of abacavir into allergenic metabolites, have also contributed to the pathogenesis of abacavir hypersensitivity (Martin et al., 2004, 2012).

Screening for *HLA-B*57:01* carriage, and using this information to preclude susceptible individuals from receiving abacavir, substantially reduced the incidence of abacavir hypersensitivity, hence relieving the costs that would have been incurred by the management of these reactions (Hughes et al., 2004a; Schackman et al., 2008). The decrease in the incidence of hypersensitivity reactions ranged from two-fold (Waters et al., 2007; Mallal et al., 2008) or four-fold (Rauch et al., 2006) to complete eradication (Zucman et al., 2007). The cost-efficiency of *HLA-B*57:01* testing is ethnicity-dependent, being largest in an all-Caucasian or a predominantly Caucasian population. Individuals of other ethnic origins, such as Africans (Hetherington et al., 2002; Hughes et al., 2004b) and Taiwanese (Sun et al., 2007), derive little benefit from such a discriminative strategy. The risk of developing abacavir hypersensitivity is considerably lower in both populations (Symonds et al., 2002; Sun et al., 2007). For instance, the incidence of abacavir-induced hypersensitivity is only 0.9% among Taiwanese individuals, coinciding with their equally rare carriage of the *HLA-B*57:01* allele (0.3% versus about 8% in Whites; Rauch et al., 2006; Sun et al., 2007). Despite the obvious influence of individual ethnic backgrounds, the Clinical Pharmacogenetics Implementation Consortium has recommended *HLA-B*57:01* screening for all patients before they are given abacavir (Martin et al., 2012, 2014). Distinguishing *HLA* alleles could be challenging, as these alleles may vary at only a few positions within their second and third exons (Robinson et al., 2015).

Use of the MinION Device to Genotype *HLA* Alleles

Several test options exist for *HLA-B*57:01* screening, with sequence-based methods being most technically complex and

deemed unsuited for routine use (Martin et al., 2012). These methods often rely on PCRs to enrich the desired *HLA* regions (Carapito et al., 2016). In a bespoke, PCR-based pipeline (Ammar et al., 2015), the *HLA-A* and *HLA-B* regions were amplified in two long-range PCRs and the resultant products (∼4 kb each) were sequenced on MinION. The accuracy of the nanopore reads was expectedly low, with the proportion of reference-discordant bases nearing 30%. The authors surmised that this could have adversely affected the genotyping results: the two samples trialed using this method were both erroneously typed. The possibility of the *HLA* haplotypes being obscured by PCR recombination or *chimerism* was alluded to but not thoroughly discussed (Ammar et al., 2015; Laver et al., 2016).

Cross-over extension or template switching is a well-known PCR artifact whereby incompletely synthesized PCR products (mega-primers) anneal to new templates giving rise to chimeric alleles (Odelberg et al., 1995). Recombined alleles would confound haplotype interpretation and it is noteworthy that such errors are clinically significant and could diminish the efficacy of MinION-centric approaches for *HLA* genotyping. For instance, in the case of characterizing the *BCR-ABL1* gene in chronic myeloid leukemia, the occurrence of PCR recombination was found to create artificial compound *BCR-ABL1* mutations, potentially misguiding anti-cancer therapy. After two rounds of PCR, totalling 80 cycles, nearly 50% of the amplicons were noted to be chimeric (Parker et al., 2014).

Several alterations could be made to the PCR protocol to attenuate template switching. For instance, the extension time could be prolonged to ensure complete synthesis of PCR products in each cycle; or the number of PCR cycles curtailed to reduce the probability of PCR recombination (McDonald et al., 2002; Laver et al., 2016). Cross-over events are more likely to occur near the end of PCR cycling or following numerous rounds of extension, during which the synthesized products are most concentrated. Alternatively, a different mechanism of enrichment could be adopted to obviate the need for these changes. Short, complementary oligonucleotides, acting as *probes*, can efficiently *capture* target *HLA* regions from a pool of genomic DNAs that have been trimmed to a pre-defined size range (Wittig et al., 2015). For MinION, it may be necessary to opt for longer probes (Karamitros and Magiorkinis, 2015) and a much less severe DNA fragmentation protocol, in order to preserve the capacity of the device to produce very long reads for the assembly of large-scale haplotypes.

Presently, a more pressing concern over MinION is perhaps its low base-calling accuracy; the error rate has frequently been estimated to exceed 10% (Check Hayden, 2015). Previous attempts at overcoming this problem have tackled chiefly two aspects of the sequencing workflow: conversion of input DNAs into sequenceable templates; and translation of ionic events into DNA bases. The first tactic derives benefit from redundant sequencing in that the input DNA is circularized and amplified in a segmental manner (rolling circle amplification) to generate tandem copies ($\geq$6) of a segment for consensus sequence determination (Li et al., 2016). This has resulted in greatly increased read accuracy (>97%) that approach those attainable by the second-generation platforms (Quail et al., 2012). However, the cost of such improved accuracy is a reduced *net* output from MinION; in other words, fewer bases are ultimately emitted per run. The second tactic is based on statistical learning enhanced by the rapidly expanding MinION datasets that are openly accessible. This has enabled mature hidden Markov models to be built for base-calling, establishing more precise event-to-sequence patterns. The construction of a sophisticated artificial neural network for DNA sequence deduction has also been suggested as a solution to the conundrum (Jain et al., 2015; Boža et al., 2016; David et al., 2016). Currently, it is uncertain which of the two strategies is superior: modified template preparation which adds some degree of complexity, or better trained *in silico* algorithms?

The relatively time-consuming nature of sequence-based *HLA* typing constitutes another limitation that must be overcome to enable routine use of the technique. For instance, it may not be uncommon for a MinION sequencing run to take one day, though it is possible to analyze the data prior to conclusion of the run. The requirement for long-range PCR amplification to isolate *HLA* genes could easily lengthen the procedure to span two days. For the synthesis of long amplicons, protracted elongation time is an inevitable bottleneck; 30–60 s are typically needed for the polymerization of ∼1000 nucleotides. A probe-based protocol may entail an even longer time of 3–4 days; but it offers the advantage of eradicating PCR chimerism by needing only 18 cycles of amplification for the enrichment of captured fragments (Karamitros and Magiorkinis, 2015; Laver et al., 2016). On the other hand, an expedited long PCR protocol may be formulated from the following ingredients: whole-blood PCR which eliminates the need for DNA extraction (Mercier et al., 1990), ultra-fast PCR empowered by prompt temperature switches (Wheeler et al., 2011), and highly processive *Taq* polymerases capable of incorporating nucleotides at a faster rate (Böhlke et al., 2000).

CONCLUSION

As we have pointed out, several issues warrant deliberation before MinION-based *HLA* typing could be considered for clinical use. The unsatisfactory data accuracy is still an unresolved issue. A modified preparatory protocol that unifies sequence information from tandem copies of a DNA segment has been shown to augment base-calling accuracy (Li et al., 2016). It may be worthwhile to compare the performance of this technique with other *in silico* approaches. The turnaround time for sequence-based tests needs to be drastically shortened; also, the testing process should be complemented by a streamlined mechanism for data analysis, interpretation, and reporting. Spartan RX, a panel indicated for the identification of CYP2C19 poor metabolizers, can generate the required result within 1 h of sample acquisition (Spartan Bioscience Inc., 2016). Above all, the actual utility of sequence-based tests begs the question as to whether the level of *HLA* genotype resolution achieved by other quicker and less laborious methods, such as allele-specific PCR, is already sufficient in the clinical setting (Martin et al., 2012). Despite these uncertainties, the diverse utility of

MinION is evident in the assortment of applications on which it has been trialed. We are optimistic that MinION will be eventually morphed into a potent tool for the diagnosis of drug hypersensitivity.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Asthma and Rhinitis Induced by Selective Immediate Reactions to Paracetamol and Non-steroidal Anti-inflammatory Drugs in Aspirin Tolerant Subjects

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In subjects with non-steroidal anti-inflammatory drugs (NSAIDs)- exacerbated respiratory disease (NERD) symptoms are triggered by acetyl salicylic acid (ASA) and other strong COX-1 inhibitors, and in some cases by weak COX-1 or by selective COX-2 inhibitors. The mechanism involved is related to prostaglandin pathway inhibition and leukotriene release. Subjects who react to a single NSAID and tolerate others are considered selective responders, and often present urticaria and/or angioedema and anaphylaxis (SNIUAA). An immunological mechanism is implicated in these reactions. However, anecdotal evidence suggests that selective responders who present respiratory airway symptoms may also exist. Our objective was to determine if subjects might develop selective responses to NSAIDs/paracetamol that manifest as upper/lower airways respiratory symptoms. For this purpose, we studied patients reporting asthma and/or rhinitis induced by paracetamol or a single NSAID that tolerated ASA. An allergological evaluation plus controlled challenge with ASA was carried out. If ASA tolerance was found, we proceeded with an oral challenge with the culprit drug. The appearance of symptoms was monitored by a clinical questionnaire and by measuring FEV1 and/or nasal airways volume changes pre and post challenge. From a total of 21 initial cases, we confirmed the appearance of nasal and/or bronchial manifestations in ten, characterized by a significant decrease in FEV1% and/or a decrease in nasal volume cavity after drug administration. All cases tolerated ASA. This shows that ASA tolerant subjects with asthma and/or rhinitis induced by paracetamol or a single NSAID without skin/systemic manifestations exist. Whether these patients represent a new clinical phenotype to be included within the current classification of hypersensitivity reactions to NSAIDs requires further investigation.

Keywords: hypersensitivity, paracetamol, non-steroidal anti-inflammatory drugs, immediate allergy, asthma, rhinitis

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most frequent elicitors of drug hypersensitivity reactions (DHRs) (Doña et al., 2012; Jares et al., 2015), being able to provoke reactions with immunological and non-immunological mechanisms (Cornejo-Garcia et al., 2009). NSAID-DHRs are now considered more frequent than betalactam (BL) DHRs, which largely induce immunologically mediated reactions (Antúnez et al., 2006b).

Since the asthma triad was first reported (Widal et al., 1993; Samter and Beers, 1968), a considerable amount of work has been dedicated to the study of ASA-induced airways disease (Pleskow et al., 1983; Szczeklik et al., 2001; Stevenson, 2004). ASA and other NSAIDs can induce various clinical entities via two major mechanisms (Kowalski et al., 2011; Asero et al., 2013; Stevenson and Kowalski, 2013). The first mechanism is pharmacological, where individuals react to chemically unrelated NSAIDs (cross intolerance), whereas the second mechanism is immune-mediated, thought to be related to specific IgE antibodies or sensitized T cells (selective reactions, SRs) (Kowalski et al., 2013). Entities in the former category are generally more frequent (Canto et al., 2009; Doña et al., 2011, 2012) and pharmacogenetic studies have been conducted for patients with respiratory airways (Ayuso et al., 2015) and cutaneous involvement (Cornejo-García et al., 2012, 2013). These studies are opening new avenues toward an understanding of the mechanisms involved (Perkins et al., 2015).

NSAIDs-exacerbated respiratory disease (NERD) belongs to the cross intolerance category and consists of chronic rhinosinusitis, nasal polyposis and/or bronchial asthma. These entities may appear in isolation but usually present together (Stevenson, 2004; Kowalski et al., 2013). They are aggravated by ASA and other strong COX-1 inhibitors, although intolerance of weak COX-1 and selective COX-2 inhibitors can also occur (Pleskow et al., 1983; Szczeklik et al., 2001; Stevenson and Kowalski, 2013). Although these patients may initially develop symptoms with different NSAIDs, a hallmark of NERD is the elicitation of a reaction after ASA intake (Asero et al., 2013; Kowalski et al., 2013).

Paracetamol is also responsible for respiratory reactions and can be a risk factor for asthma (Blanca-López et al., 2015) development, although the underlying mechanisms are currently unknown (Lesko, 2002).

Few studies have examined individuals who react to a single NSAID or paracetamol but tolerate ASA (Corominas et al., 2012). Nevertheless, the existence of this type of response has been shown (Cavkayter et al., 2015). One of the problems when studying this entity is that respiratory airways involvement may occur during an anaphylactic reaction to a single NSAID (Doña et al., 2011; Ayuso et al., 2013).

In this study, we present a series of cases who reported isolated upper and/or lower respiratory symptoms after taking a COX-1 or selective COX-2 inhibitor, but tolerated other NSAIDs including ASA. Diagnosis was confirmed by controlled challenge with the culprit drug and proving ASA tolerance.

MATERIALS AND METHODS

Patient Selection

Patients reporting a respiratory reaction but no other symptoms after NSAIDs or paracetamol intake were evaluated in two centers integrated into the Spanish National Network for the Study of Drug and Allergen Hypersensitivity (Infanta Leonor University Hospital in Madrid, and Regional University Hospital of Malaga). A prerequisite for inclusion was that the patient reported ASA tolerance – this was then confirmed by ASA challenge in this study. In addition, challenge with the culprit drug was performed to confirm the diagnosis of a selective respiratory reaction (Doña et al., 2011, 2012). We did not include subjects with chronic spontaneous urticaria, nasal polyposis, respiratory infections, autoimmune diseases, pregnancy or who were undergoing breast-feeding.

This study was performed according to the principles of the Declaration of Helsinki and approved by the local ethics committee from the Infanta Leonor University Hospital and the Regional University Hospital of Malaga. All patients were informed orally about the study and signed the corresponding informed consent.

Clinical Evaluation

Patients were subjected to a clinical questionnaire, skin prick testing (SPT) and had total serum IgE levels measured. Nasal and bronchial symptoms were assessed as described (Doña et al., 2011; Campo et al., 2013). Symptoms attributable to foods, hymenoptera sting and potential drug allergies were also recorded. A panel of 30 common inhalant and food allergens prevalent in the area of study was used for skin testing. A wheal diameter larger than 3 mm was considered positive. A negative saline control was also used.

Nasal acoustic rhinomanometry was performed and basal FEV1 estimated (Campo et al., 2013). Nasal exploration including a CT scan was also performed to exclude patients with polyposis, chronic rhinosinusitis or any other underlying nasal disease.

Nasal Challenge with Lysine-Aspirin (lys-ASA)

Nasal challenge with lys-ASA was performed as described (Lee et al., 2004; Nizankowska-Mogilnicka et al., 2007). The appearance of nasal symptoms plus a decrease of 30% in the total volume of both nasal cavities measured by acoustic rhynometry (AR) was considered a positive result. The response of the lower airways after nasal challenge was also monitored by FEV1. A decrease greater than 12% compared to basal values was considered positive (Campo et al., 2013).

Oral Provocation with ASA

This was performed using a single blind procedure as described (Doña et al., 2011), with the following modifications: on the first day, we administered placebo and if no reaction occurred, increasing oral concentrations of ASA were given at intervals of 90 min (doses of 25, 100, and 250 mg; cumulative dose of 375 mg). If no symptoms appeared, we repeated the challenge 1 week later

but with a starting dose of 500 mg of ASA; if this was tolerated another 500 mg dose was given 90 min later (cumulative dose of 1000 mg).

Oral Provocation with the Culprit Drug

When using the suspected culprit drug, we started with lower concentrations, following previous recommendations (Doña et al., 2011). For ibuprofen, increasing doses of 5, 20, 75, 150, and 350 mg were given at 45-minute intervals. If symptoms appeared at any time the procedure was stopped immediately. Clinical symptoms were assessed and changes in nasal flow were monitored by acoustic rhynomanometry; lower airways involvement was monitored by FEV1. For paracetamol the same approach was used but with doses of 5, 20, 50, 75, 150, and 200 mg. If symptoms did not appear, another 500 mg of paracetamol were given (cumulative dose of 1000 mg). For rofecoxib, we used doses of 5, 15, 40, and 60 mg (cumulative dose of 120 mg), whereas for dexketoprofen, we used doses of 5, 20, and 25 mg (cumulative dose of 50 mg).

RESULTS

A total of 21 patients reporting symptoms of rhinitis and/or asthma after the intake of paracetamol or a single NSAID, but also reporting ASA tolerance were prospectively recruited from January 2010 to December 2014. In all patients ASA tolerance was also assessed by challenge as described in the Methods section.

After completing the study, nasal and/or lower airways symptoms attributable to the intake of the culprit drug could be confirmed for 10 patients (**Table 1**). There was no clear difference in gender (four were female; six male). Their ages ranged from 22 to 69 years old, with a mean of 51.5. In terms of atopy, six were atopic and four non-atopic. The mean number of episodes was 4.3, although this value ranged from 2 to 10 amongst patients.

Ibuprofen was involved in four cases, paracetamol in 4, desketoprofen in 1 and etoricoxib in 1 (**Table 1**). Most patients reported three or more previous episodes (except patients 7 and 10, who reported only 2 previous episodes). Rhinitis, with or without asthma, appeared in six patients whereas isolated asthma occurred in four. Concerning atopic status, six patients showed a positive SPT to more than one common inhalant allergen (**Table 1**). No positive SPT to food allergens was observed. Clinical entities and the time interval elapsed between drug intake and appearance of symptoms according to patient history are provided in **Table 1**, whereas **Table 2** shows the challenge results.

Although statistical comparisons were not performed due to the limited number of patients, we observed a tendency for ibuprofen to induce a reaction at higher doses and after longer time intervals compared to paracetamol (**Table 2**).

Concerning the cumulative dose required to elicit the reaction, this was variable for each drug (**Table 2**). For three patients the response to ibuprofen appeared at a dose of less than half the therapeutic dose; this also occurred in three patients who reacted to paracetamol.

DISCUSSION

In this study, we have presented for the first time a series of patients with SRs to paracetamol or a single NSAID with exclusive respiratory airway involvement. The approach for identifying these patients was based on clinical history, negative challenge with ASA and positive challenge with the culprit drug. Of the 21 cases initially considered, ten could be confirmed as tolerating asthma but reacting to the culprit drug. On average,

TABLE 1 | Clinical characteristics and drugs involved.

Patient No.	Sex	Age (years)	Atopy	Positive allergen SPT	Serum specific IgE (kU/l)	Culprit drug	No. of episodes	Time interval (min)	Reaction
1	F	47	+	*Phleum pratense* *Lollium perenne*	2.15 1.65	Dexketoprofen	3	20–30	Asthma
2	F	22	+	*Lollium perenne*	1.55	Etoricoxib	3	30–60	Rhinitis
3	F	69	+	*Alternaria alternata* *Aspergillus fumigatus*	1.31 11.7	Ibuprofen	6	30–60	Rhinitis
4	M	38	+	*Dermatophagoides pteronyssinus* *Olea europaea*	1.25 1.95	Ibuprofen	7	60–120	Rhinitis + Asthma
5	M	65	–	NA	NA	Ibuprofen	3	60–120	Rhinitis + Asthma
6	F	47	+	*Dermatophagoides pteronyssinus* *Alternaria alternata* *Aspergillus fumigatus*	2.34 1.25 1.45	Ibuprofen	4	60–120	Rhinitis + Asthma
7	M	66	–	NA	NA	Paracetamol	2	20–30	Asthma
8	M	50	+	*Olea europaea* *Lollium perenne* Cat dander	1.23 3.23 1.60	Paracetamol	10	20–30	Asthma
9	M	35	–	NA	NA	Paracetamol	3	30–60	Asthma
10	M	56	–	NA	NA	Paracetamol	2	30–60	Rhinitis

NA, not applicable.

TABLE 2 | Results of challenge: time intervals between drug administration and the appearance of clinical symptoms, last and cumulative doses, and clinical symptoms induced.

Patient No.	Culprit drug	No. of episodes	Time interval (min)	Reaction
1	Dexketoprofen	3	20–30	Asthma
2	Etoricoxib	3	30–60	Rhinitis
3	Ibuprofen	6	30–60	Rhinitis
4	Ibuprofen	7	60–120	Rhinitis + Asthma
5	Ibuprofen	3	60–120	Rhinitis + Asthma
6	Ibuprofen	4	60–120	Rhinitis + Asthma
7	Paracetamol	2	20–30	Asthma
8	Paracetamol	10	20–30	Asthma
9	Paracetamol	3	30–60	Asthma
10	Paracetamol	2	30–60	Rhinitis

AR, acoustic rhynometry; FEV1, forced expiratory volume in 1 s; DPT, drug provocation test.

these patients were older than the eleven other cases and reported more previous episodes.

Selective reactions to NSAIDs have been reported by several groups and for all available NSAIDs and selective COX-2 inhibitors (reviewed in Canto et al., 2009; Cornejo-Garcia et al., 2009; Blanca-Lopez et al., 2014; Torres et al., 2014). Pyrazolones, although not considered NSAIDs, are common triggers (Kowalski et al., 2013; Demir et al., 2015); other important drugs include diclofenac (Gala et al., 1998; Del Pozo et al., 2000; Harrer et al., 2010; Picaud et al., 2014), ibuprofen (Koransky et al., 2016), as well as weak COX-1 (Vidal et al., 1997; Astarita et al., 2011), and COX-2 inhibitors (Fontaine et al., 2005; Chamberlin and Silverman, 2009). In fact, in some countries SRs are responsible for up to 50% of all NSAID-DHRs (Demir et al., 2015). However, in these reported cases the symptoms induced were anaphylaxis and/or urticaria, and in those reactions with respiratory airway involvement, other organs were also implicated (Doña et al., 2011). All patients included in this study developed respiratory symptoms only, i.e., nasal symptoms and/or AR/FEV1 decreases, without other organ involvement.

For pyrazolones, positive skin and/or basophil activation test results have been found with metamizole (Gomez et al., 2009), providing indirect evidence of a potential IgE-mediated reaction. However, the only pyrazolone for which specific IgE-mediated reactions have been demonstrated is propyphenazone (Himly et al., 2003). Positive skin tests results for other NSAIDs are almost anecdotal with very few cases reported (Canto et al., 2009). Consequently, we did not perform skin testing with the culprit drug here.

Considering cross intolerance reactions, patients may present both respiratory and cutaneous involvement (blended reactions) (Stevenson and Kowalski, 2013), which have been reported as being more frequent than cases with exclusively respiratory symptoms (Doña et al., 2011). Questions remain as to whether blended reactions constitute a well-defined entity or a more heterogeneous group. Our feeling is that this entity includes different phenotypes (Ayuso et al., 2013).

Concerning respiratory SRs, only one case has been reported in the literature so far (Corominas et al., 2012). Repeated episodes of bronchospasm induced by paracetamol occurred in a 19-year-old atopic woman, who tolerated 500 mg of ASA. After the intake of 500 mg of paracetamol she presented a severe episode of bronchospasm with a decrease in FEV1 that required treatment with salbutamol (Corominas et al., 2012). Although the existence of respiratory SRs has been suggested previously (Cavkayter et al., 2015), this pattern of reaction has not been clearly identified. The identification of these reactions may be complicated due to respiratory symptoms being accompanied with mild symptoms in other organs: these reactions may be hard to distinguish from anaphylactic reactions. In fact, in IgE-mediated DHRs an initial mild reaction can be followed by a more severe one after subsequent exposure (Antúnez et al., 2006a). This phenomenon is thought to be due to greater levels of IgE antibodies and increased affinity. For the patients reported here, no evidence for increasing reaction severity was found.

Summarizing, in this study, we have presented a group of patients with an unusual pattern of response, which does not fit properly in the current classification system of DHRs to NSAIDs (Ayuso et al., 2013; Kowalski et al., 2013; Agúndez et al., 2015). Further attempts are underway to identify more patients with these characteristics and potentially establish the mechanisms involved.

AUTHOR CONTRIBUTIONS

NB-L, JC-G, MB, and GC conceived the study, revised protocols and data, and wrote the manuscript. DP-A, MB, NB-L, and ID selected all patients, performed the allergological workup and analyzed data. JA, EG-M, and JP analyzed data and revised the manuscript. JP reviewed the final English version. All the authors read and approved the final manuscript.

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MINI REVIEW
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Biomarkers in DILI: One More Step Forward

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Despite being relatively rare, drug-induced liver injury (DILI) is a serious condition, both for the individual patient due to the risk of acute liver failure, and for the drug development industry and regulatory agencies due to associations with drug development attritions, black box warnings, and postmarketing withdrawals. A major limitation in DILI diagnosis and prediction is the current lack of specific biomarkers. Despite refined usage of traditional liver biomarkers in DILI, reliable disease outcome predictions are still difficult to make. These limitations have driven the growing interest in developing new more sensitive and specific DILI biomarkers, which can improve early DILI prediction, diagnosis, and course of action. Several promising DILI biomarker candidates have been discovered to date, including mechanistic-based biomarker candidates such as glutamate dehydrogenase, high-mobility group box 1 protein and keratin-18, which can also provide information on the injury mechanism of different causative agents. Furthermore, microRNAs have received much attention lately as potential non-invasive DILI biomarker candidates, in particular miR-122. Advances in "omics" technologies offer a new approach for biomarker exploration studies. The ability to screen a large number of molecules (e.g., metabolites, proteins, or DNA) simultaneously enables the identification of 'toxicity signatures,' which may be used to enhance preclinical safety assessments and disease diagnostics. Omics-based studies can also provide information on the underlying mechanisms of distinct forms of DILI that may further facilitate the identification of early diagnostic biomarkers and safer implementation of personalized medicine. In this review, we summarize recent advances in the area of DILI biomarker studies.

Keywords: hepatotoxicity, drug-induced liver injury, prediction, diagnosis, outcome

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INTRODUCTION

Drug-induced liver injury (DILI) is one of many forms of adverse drug reactions that appear in a small proportion of patients. DILI is generally classified as intrinsic if predictable based on dose and pharmacological properties [for example acetaminophen (APAP) overdose] or idiosyncratic when unpredictable by the same features. The latter is believed to be a consequence of interactions between drug properties, host factors, and environmental conditions in a susceptible individual. Hence, no functional animal models are currently available for idiosyncratic DILI, unlike the presence of well-established murine models for APAP hepatotoxicity. DILI can have a profound

impact on patient health due to its potential to cause acute liver failure, although most idiosyncratic DILI cases have a favorable outcome with full recovery after withdrawal of the culprit drug. DILI presents a clinical challenge as it can mimic almost any acute or chronic hepatobiliary condition and there are currently no specific biomarkers or diagnostic test available for this condition. A clinical diagnosis of idiosyncratic DILI therefore relies heavily on exclusion of alternative causes and a compatible drug history. The lack of DILI specific biomarkers also affects the drug development process and can result in early termination of drug candidates with assumed idiosyncratic hepatotoxicity potential. This can have considerable economic consequences and can prevent a large targeted recipient group from benefitting from a drug that may only be harmful to a small proportion of patients. New more specific and sensitive DILI biomarkers could enable better monitoring of patients receiving new drugs and minimize liver injury through early detection and subsequent cessation of dosing.

CURRENT BIOMARKERS IN DILI

Diagnosis

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). While of uttermost importance in preclinical and early-phase clinical trial efficacy and safety evaluations of new drug compounds, biomarkers also play important roles in clinical practice to diagnose specific diseases, determine the state of a disease, disease prognosis and monitoring of clinical response to an intervention. Due to the lack of specific DILI biomarkers, DILI is monitored and diagnosed based on general liver injury serum biomarkers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TBL). ALT, AST, and ALP are all intracellular enzymes, which when detected in serum can indicate injury to hepatocytes or biliary cells. An increase in serum TBL on the other hand reflects liver dysfunction, but while being liver-specific it is relatively insensitive and subsequently appears later along the disease progression. ALT, AST, and ALP are not liver-specific and extrahepatic conditions such as myocardial damage, skeletal muscle diseases, hyperthyroidism, and bone diseases are associated with increased ALT, AST, or ALP. Interestingly, evidence of ALT and ALP plasma levels being influenced by genetic variations are also emerging (Yuan et al., 2008).

Alanine aminotransferase is thought to be more specific for liver injury than AST, as it is present mainly as a cytosolic protein in the liver and in low concentrations elsewhere, while AST is present in both cytosolic and mitochondrial forms in liver, heart, skeletal muscle, kidney, brain, pancreas, and lung tissue as well as in white and red blood cells (Giboney, 2005). Human ALT, however, exists as two isoforms with identical enzyme capacity, ALT1 and ALT2, although the presence of ALT2 appears to be restricted to non-hepatic tissue, in particular heart and skeletal muscles (Glinghammar et al., 2009). A recent study has demonstrated that assessing the percentage contribution of ALT1 and ALT2 activities to total ALT activity in plasma may distinguish hepatic from extrahepatic injury (Rafter et al., 2012). The standard ALT activity assay currently used in clinical practice, however, does not discriminate between serum ALT from different organs or isoforms

Disease Phenotype

Drug-induced liver injury is generally classified into three patterns of liver injury (hepatocellular, cholestatic, and mixed), based originally on histological features. Hepatocellular type of injury is characterized by hepatocyte necrosis and inflammation, while bile stasis, portal inflammation and bile duct injury are commonly found in cholestatic injury. Mixed injury is, as the name suggests, a combination of these two liver injury types. As biopsies not normally form part of the DILI diagnostic process, the pattern of injury is mainly deduced based on liver profile analytical values. More precisely, the relationship between ALT and ALP values at DILI onset are used to determine the type of liver injury according to the R formula, R = (ALT/upper limit of normal, ULN) ÷ (ALP/ULN; Benichou, 1990; Aithal et al., 2011). An R value $\geq$ 5 is indicative of hepatocellular damage, R $\leq$ 2 cholestatic damage and 2 $<$ R $<$ 5 mixed damage. A recent comparison of ALT and AST elevations in a large DILI cohort found that AST can reliably substitute for ALT in R value calculations in cases were ALT values are absent at DILI onset, but that gamma-glutamyl transpeptidase (GGT) is less reliable as an ALP substitute (Robles-Diaz et al., 2015).

Clinical Course and Prognosis

An ALT value alone offers little predictive value with regards to the clinical course of DILI, but can provide a prognostic indication when combined with TBL. This is today referred to as Hy's Law and is based on clinical observations made by the late Hyman Zimmerman that DILI patients with hepatocellular type of liver injury and jaundice have a 10–50% higher risk of mortality or need for liver transplantation. Hy's Law has since been defined more specifically as AST or ALT $>$ 3 xULN and TBL $>$ 2 xULN in the absence of ALP elevations and alternative reasons for ALT and TBL elevations, and is endorsed by the American Food and Drug Administration as criteria for severe DILI[1].

The validity of Hy's Law has now been confirmed in various large DILI cohorts (Andrade et al., 2005; Björnsson and Olsson, 2005; Chalasani et al., 2015). However, the effect of ALP increases on Hy's Law have been questioned and recent findings demonstrate that an ALP level greater than 2 xULN does not have a protective role in acute liver failure development (Robles-Diaz et al., 2014). While having relatively high sensitivity, Hy's Law lacks specificity as the majority of DILI cases meeting the Hy's Law criteria in fact have a favorable outcome with full recovery. Using an extended range of analytical values we have developed a composite algorithm with higher specificity for the prediction of acute liver failure based on DILI onset data (Robles-Diaz et al., 2014).

[1] http://www.fda.gov/downloads/Drugs/.../Guidances/UCM174090.pdf

TABLE 1 | New serum/plasma DILI biomarker candidates.

Biomarker	Study cohort (N)	Comparison to alanine aminotransferase (ALT)	Reference
GLDH	Human: APAP overdose (33), cirrhosis and liver injury (108), hepatic carcinoma (40)	Overall correlation coefficient: 0.88. Lower correlation in APAP overdose than other liver injuries	Schomaker et al., 2013
GLDH	Human: APAP overdose (129)	Correlation coefficient (peak ALT): 0.45	Antoine et al., 2013
GLDH	Human: healthy volunteers receiving heparin	Correlation coefficient (peak ALT): 0.76	Harrill et al., 2012
MDH	Human: APAP overdose (33), cirrhosis and liver injury (108), hepatic carcinoma (40)	Overall correlation coefficient: 0.74. Less liver-specific than ALT.	Schomaker et al., 2013
HMGB1	Mouse: APAP overdose	Detectable elevations prior to ALT elevations. Peak value reached faster than ALT.	Antoine et al., 2009
HMGB1	Human: APAP overdose (84)	Correlation coefficient: 0.60. Acetylated HMGB1 associated with worse prognosis	Antoine et al., 2012
HMGB1	Human: APAP overdose (129)	Correlation coefficient (peak ALT): 0.67. Can predict clinical hepatotoxicity after APAP overdose prior to ALT.	Antoine et al., 2013
K18	Mouse: APAP overdose	Detectable elevations of FL-K18 and cK18 prior to ALT.	Antoine et al., 2009
K18	Human: APAP overdose (84)	Correlation coefficient: 0.58. Full length K18 associated with worse prognosis	Antoine et al., 2012
K18	Human: APAP overdose (129)	Correlation coefficient (peak ALT): FL-K18, 0.59; cK18, 0.57. FL-K18 can predict clinical hepatotoxicity after APAP overdose prior to ALT.	Antoine et al., 2013
K18	Humans: healthy volunteers receiving APAP (58), participating in an extreme adventure race (12)	Correlation coefficient (peak ALT): FL-K18, 0.70; cK18, 0.66. No elevation after muscular injury, which increased ALT	Thulin et al., 2014
miR-122, mir-192	Mouse: APAP overdose	Liver enriched miRs. Dose- and exposure duration-dependent changes in plasma detectable earlier than ALT.	Wang et al., 2014
miR-122, miR-192	Humans: APAP overdose (53)	Increased serum level after APAP overdose. miR-122 correlation coefficient (peak ALT): 0.46.	Starkey Lewis et al., 2011
miR-122	Humans: healthy volunteers receiving APAP (58), participating in an extreme adventure race (12)	Correlation coefficient (peak ALT): 0.62. No elevation after muscular injury, which increased ALT	Thulin et al., 2014
Eleven miRNAs profile	Humans: APAP overdose and ischemic hepatitis (49)	Diagnostic potential (elevated in APAP overdose but not in ischemic patients). Lack of miRNA profile recovery indicative of adverse patient outcome.	Ward et al., 2014

GLDH, glutamate dehydrogenase; MDH, malate dehydrogenase; HMGB1, high-mobility group box-1 protein; K18, keratin-18; FL-K18, full length K18; cK18, caspase-cleaved fragment of K18; miR, microRNA.

NEW POTENTIAL BIOMARKERS IN DILI

The heightened interest in developing new more sensitive and specific DILI biomarkers for early prediction and diagnosis[2,3,4] has resulted in the discovery of several promising candidates (**Table 1**). Many of these biomarker candidates were initially identified using APAP models, but could also be promising for aspects of idiosyncratic DILI.

Mechanistic-Based

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme found primarily in the liver and to a lesser degree in kidneys and trace amounts in skeletal muscles. This enzyme is more tissue-specific than ALT and AST. Healthy individuals present a stable and measurable level of serum GLDH that is unaffected by age and gender (Schomaker et al., 2013). A rise in circulating GLDH indicates mitochondrial dysfunction and subsequent loss of mitochondrial membrane integrity, which occurs during hepatocellular necrosis. GLDH correlates well with ALT elevations in patients with various forms of clinically demonstrated liver injury, including APAP overdose, but is not sensitive enough to predict APAP hepatotoxicity prior to ALT (Antoine et al., 2013; Schomaker et al., 2013). Furthermore, healthy volunteers receiving subcutaneous heparin injection have been reported to develop serum GLDH elevations in conjunction with asymptomatic ALT elevations, which underline the correlation between GLDH and ALT (Harrill et al., 2012). Similar results have been found for malate dehydrogenase (MDH), a constitutive enzyme in the citric acid cycle that is released into the serum after tissue damage (Schomaker et al., 2013). However, MDH is less tissue-specific and its serum level is subsequently affected by extrahepatic tissue damage.

High-mobility group box 1 (HMGB1) is a chromatin-binding protein with proinflammatory activity mediated through Toll-like receptor and receptor for advanced glycation endproducts signaling (Kubes and Mehal, 2012). It is passively released by necrotic cells in a hypoacetylated form, but not by apoptotic or secondary necrotic cells (Scaffidi et al., 2002). In contrast,

[2]http://www.imi-safe-t.eu/

[3]http://www.mip-dili.eu

[4]https://c-path.org/programs/pstc/

it is actively secreted in a hyperacetylated form by immune cells (Bonaldi et al., 2003). Hence, HMGB1 is reflective of cell necrosis or activated immune cells depending on its acetylation status. Keratin-18 (K18) is a member of the keratin protein family, which is involved in cell structure and integrity. Similar to HMGB1, full-length K18 is released passively during cell necrosis. However, K18 undergoes caspase-mediated cleavage as part of structural rearrangements during apoptosis and can then be released into the blood stream (Ku et al., 2007). Hence, differentiating serum concentrations of full-length and caspase-cleaved K18 can provide an indication of the level of cell necrosis and apoptosis occurring in a subject. The molecular forms of HMGB1 and K18 have now been verified as sensitive blood-based markers of acute liver injury and provide insight into the mechanistic process of APAP hepatotoxicity (Antoine et al., 2009, 2012). These biomarkers have also been found to be more sensitive than ALT, with apparent elevations prior to ALT in patients at first presentation after APAP overdose intake (Antoine et al., 2013). The use of these biomarkers in clinical practice could potentially improve the speed of clinical decision-making. However, the value and clinical utility in cases with idiosyncratic DILI is still unknown.

MicroRNA

MicroRNAs (miRNAs) are small (20–24 nucleotides) non-coding RNAs involved in post-transcriptional regulation of gene expression. Organ damage usually results in miRNAs being released into the bloodstream and to some degree also in urine. MiRNAs are relatively stable in biofluids, a feature that has contributed to that circulating miRNAs have received much attention lately as potential non-invasive DILI biomarker candidates. To date several studies have reported changes in serum miRNA concentrations during liver injury (Zhang et al., 2010; Starkey Lewis et al., 2011; Ward et al., 2014). MiR-122 and miR-192 were the first circulating miRNAs demonstrated to increase after toxic APAP doses in mice and soon after were confirmed to behave in a similar manner in human subjects (Wang et al., 2009; Starkey Lewis et al., 2011). MiR-122 and miR-192 are both liver-enriched miRNAs, with miR-122 variants accounting for approximately 72% of the total liver miRNA population in mice (Lagos-Quintana et al., 2002). Serum miR-122 is detectable at an early stage of hepatocellular damage as demonstrated in hospital admitted patients shortly after APAP overdose intake when ALT still remained normal (Antoine et al., 2013). This feature makes miR-122 particularly interesting for use during early phase human trials to detect drug candidates with hepatotoxicity potential, which may go undetected with the current biomarkers. Furthermore, miR-122 is more liver-specific than ALT as demonstrated in a human cohort with exercise-induced muscular injury, in which miR-122 remained stable while increases in ALT were detected (Thulin et al., 2014).

Emerging data also indicate that miRNA-122 could have a prognostic value, with higher early serum levels reported in APAP overdose patients who met the King's College Criteria for liver transplantation (Starkey Lewis et al., 2011). However, the value of miR-122 for idiosyncratic DILI remains to be determined. It is interesting to note that while miR-122 shows much promise as a sensitive early blood borne DILI biomarker, urine levels of this miRNA does not increase notably after APAP overdoses neither in rats nor in humans (Yang et al., 2012, 2015). Recent data also suggest that serum miRNA profiles, rather than individual miRNAs, could have a higher diagnostic value. This is supported by a comparison of an 11-miRNA panel in acute liver injury patients of different etiologies (Ward et al., 2014).

Exosomes

While miRNAs can enter the blood stream passively during cell necrotic and/or apoptotic cell death, active release also occurs in a regulated manner via exosomes. Exosomes are one of several forms of membrane-surrounded structures released by almost all types of cells. The complete role of exosomes besides transportation and delivery of various substances, such as signaling molecules and cellular waste, is yet to be elucidated (Yang et al., 2014). The constituents of exosomes are not limited to miRNAs but also include proteins, lipids, and additional nucleic acids (mRNAs). Exosomes have received much attention lately as a potential source for DILI biomarker explorations, seeing that exosome constituents change under cellular stress conditions (Wetmore et al., 2010; de Jong et al., 2012). Circulating exosomes originating from various tissues are found in many types of body fluids, with blood and urine being the more interesting sources for non-invasive biomarkers. A major challenge is subsequently to determine hepatocyte-derived exosomes. Nevertheless, promising results have emerged using animal models to demonstrate increases in exosomal liver-specific mRNAs (e.g., albumin, fibrinogen B β-polypeptide, haptoglobin, and β-actin) and liver-enriched miRNAs (miR-122 and miR-155) during acute liver injury (Wetmore et al., 2010; Bala et al., 2012). Interestingly, while increased levels of miR-122 and miR-155 were detected in circulating exosome-rich serum/plasma fractions in mice with alcohol- or lipopolysaccharide-induced liver injury, these miRNAs were found to be elevated in protein-rich serum/plasma fractions in mice with APAP hepatotoxicity, suggesting that miRNA compartment distribution pattern could also differ depending on the etiology (Bala et al., 2012). Variations in exosome-derived miRNA expression profiles have also been reported for additional liver conditions, such as chronic viral hepatitis B and C and non-alcoholic steatohepatitis compared to healthy controls. Furthermore, the miRNA expression pattern was found to reflect fibrosis stage and grade of liver inflammation in patients with chronic viral hepatitis C (Murakami et al., 2012).

"Omics" Derived Biomarkers

The introduction of omics technologies offers a new approach for biomarker exploration studies. The ability to screen a large number of molecules (metabolites, proteins, DNA, etc.) simultaneously enables the identification of 'toxicity signatures', which could be used to enhance preclinical safety assessments and disease diagnostics. Omics-based studies can also provide information on the underlying mechanisms of distinct forms of DILI that could further facilitate the identification of early diagnostic biomarkers. Metabolomics is recognized as a promising technique, typically through mass spectrometry or

nuclear magnetic resonance spectroscopy. This technique enables the evaluation of global metabolic changes and subsequently the identification of specific metabolic profiles associated with for example diseases or treatment responses. Metabolomics has been successfully applied to identify potential biomarker candidates in many types of liver diseases, including non-alcoholic fatty liver disease, steatosis, fibrosis, cirrhosis, hepatocellular carcinoma, and cholangiocarcinoma (reviewed by Beyoğlu and Idle, 2013). Furthermore, a comparison of plasma metabolic profiles of patients diagnosed with autoimmune hepatitis, primary biliary cirrhosis, autoimmune hepatitis/primary biliary cirrhosis overlap syndrome, DILI and healthy controls resulted in a metabolic autoimmune hepatitis phenotype with >93% sensitivity and specificity (Wang et al., 2014). Similarly, serum γ-glutamyl dipeptide levels have been demonstrated as potential biomarkers capable of discriminating between different forms of hepatic diseases (Soga et al., 2011).

However, in the field of hepatotoxicity, metabolomics studies performed to date are mainly limited to intrinsic DILI in animal or *in vitro* models. An ultra-performance liquid chromatography/time-of-flight tandem mass spectrometry analysis of galactosamine-treated rat sera found differences in several metabolites such as glucose, amino acids, and membrane lipids compared with control rats, with some of these correlating with the degree of histologically determined liver damage (Gonzalez et al., 2012). A similar study of sera from drug-treated rats found increases in bile acid levels in addition to several other metabolite differences, which combined with conventional clinical chemistry markers may improve the robustness and accuracy of a hepatotoxicity diagnosis (Buness et al., 2014). Recent animal studies also support that metabolic profiles, rather than individual metabolites, can predict hepatotoxicity, including idiosyncratic hepatotoxicity (Sun et al., 2014; Li et al., 2016). Metabolic profiling in urine has also been performed in search for metabolites associated with DILI. Healthy volunteers administered non-toxic levels of APAP presented both urine and plasma metabolic alterations when comparing pre- and post-dosing samples, which were more sensitive than changes in serum biochemical parameter over the study period (Kim et al., 2013). It should be pointed out that while many metabolic studies to date coincide in detecting variations in endogenous metabolites, the identified metabolites often vary between studies. Differences in study design as well as treatments could lead to such variations. It is also important to keep in mind that interindividual differences are common in metabolomics due to this method being very sensitive and subsequently can reflect variations in environmental and host conditions between subjects. Nevertheless, metabolomics could be a practical method for identifying DILI patients shortly after starting a new drug treatment and subsequently minimize serious liver injury through early treatment discontinuation when specific metabolic changes are detected. In addition to metabolomics, proteomics can be useful in the search for more specific DILI biomarkers. An exploratory comparison of global serum proteomes in DILI has reported promising results with apolipoprotein E expression demonstrating the greatest power to differentiate DILI from controls (Bell et al., 2012), while cadherin 5 and fatty acid binding protein 1 were found to be associated with DILI using an antibody bead array approach (Mikus et al., 2016). Likewise, genome-wide association studies of DILI cohorts have provided a number of specific HLA alleles that appear to be associated with distinct forms of DILI. These alleles, which have high negative predictive values, may be used as biomarkers to rule out DILI or identify the correct causative agent in patients taking more than one hepatotoxic agent, and subsequently facilitate the DILI diagnostic process (Aithal, 2015). However, the DILI forms associated with HLA risk alleles to date are limited to more common causative agents and many forms of DILI consequently lack determined HLA associations.

FUTURE PERSPECTIVES

Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin are currently the only approved DILI biomarkers in clinical practice. While fulfilling an important role in disease diagnostics, these biomarkers are not specific for hepatotoxicity, as an increase in any of these biochemical values is detected during practically all liver conditions. Furthermore, these biomarkers lack sensitivity as they appear once liver damage has developed and are consequently of limited use for predicting potential liver injury at a very early stage. This is of particular importance in clinical trials during drug development. The presence of DILI biomarkers with enhanced specificity and sensitivity may detect drug candidates with hepatotoxicity potential earlier in the drug development process and subsequently reduce the number of late stage drug attrition and postmarketing drug withdrawals. Furthermore, such biomarker would improve patient safety, due to offering improved monitoring during drug treatment initiation, as well as a faster and more accurate DILI diagnosis.

The lack of specific DILI biomarkers is to a large extent a consequence of the limited mechanistic understanding of this condition. The arrival of the "omics era" has brought hope and anticipation of scientific breakthroughs on the mechanistic side of DILI. Substantial progress has been made recently with the identification of new mechanistic-based biomarker candidates, such as HMGB1, K18, and miR-122. However, further efforts to explore and refine new improved DILI biomarker panels are needed. To be valid in clinical practice such non-invasive biomarkers not only need to demonstrate substantial specificity and sensitivity, but also need to be reasonably stable in extracted body fluids to allow reliable detection, as well as being easily assayed. The ability to validate new DILI biomarker candidates is restricted due to the current lack of fully functional animal models for idiosyncratic DILI. Although improved cell culture system such as induced pluripotent stem cell-derived hepatocytes are emerging, large DILI patient cohorts are the foremost approach for biomarker validations. This highlights the importance of collaborative efforts to establish DILI registries, which can circumvent the low DILI incidence rate generally encountered in individual hospital units. The Pro-Euro-DILI Registry is a recently established international DILI registry with serial biological sample collections from DILI onset to

normalization of enrolled patients, intended for future DILI biomarker studies and validations (Slim et al., 2016).

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Photosensitivity to Triflusal: Formation of a Photoadduct with Ubiquitin Demonstrated by Photophysical and Proteomic Techniques

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Triflusal is a platelet aggregation inhibitor chemically related to acetylsalicylic acid, which is used for the prevention and/or treatment of vascular thromboembolisms, which acts as a prodrug. Actually, after oral administration it is absorbed primarily in the small intestine, binds to plasma proteins (99%) and is rapidly biotransformed in the liver into its deacetylated active metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB). In healthy humans, the half-life of triflusal is *ca.* 0.5 h, whereas for HTB it is *ca*. 35 h. From a pharmacological point of view, it is interesting to note that HTB is itself highly active as a platelet anti-aggregant agent. Indeed, studies on the clinical profile of both drug and metabolite have shown no significant differences between them. It has been evidenced that HTB displays ability to induce photoallergy in humans. This phenomenon involves a cell-mediated immune response, which is initiated by covalent binding of a light-activated photosensitizer (or a species derived therefrom) to a protein. In this context, small proteins like ubiquitin could be appropriate models for investigating covalent binding by means of MS/MS and peptide fingerprint analysis. In previous work, it was shown that HTB forms covalent photoadducts with isolated lysine. Interestingly, ubiquitin contains seven lysine residues that could be modified by a similar reaction. With this background, the aim of the present work is to explore adduct formation between the triflusal metabolite and ubiquitin as model protein upon sunlight irradiation, combining proteomic and photophysical (fluorescence and laser flash photolysis) techniques. Photophysical and proteomic analysis demonstrates monoadduct formation as the major outcome of the reaction. Interestingly, addition can take place at any of the ε-amino groups of the lysine residues of the protein and involves replacement of the trifluoromethyl moiety with a new amide function. This process can in principle occur with other trifluoroaromatic compounds and may be responsible for the appearance of undesired photoallergic side effects.

Keywords: covalent binding to protein, fluorescence, laser flash photolysis, lysine, mass spectrometry, metabolite, photoallergy

INTRODUCTION

Triflusal (**Figure 1**) is a platelet aggregation inhibitor chemically related to acetylsalicylic acid, which is used for the prevention and/or treatment of vascular thromboembolisms (McNeely and Goa, 1998; Gonzalez-Correa and De La Cruz, 2006). Additionally, triflusal increases nitric oxide synthesis in neutrophils, leading to an increased vasodilator potential (Matías-Guiu et al., 2003).

From a clinical standpoint, triflusal shows similar efficacy to aspirin in preventing stroke, but the former is associated with a reduced risk of hemorrhagic complications. Structurally, it differs from the latter in having a trifluoromethyl moiety at position 4 (Matías-Guiu et al., 2003; Gonzalez-Correa and De La Cruz, 2006).

Actually, triflusal acts as a prodrug since after oral administration, it is absorbed primarily in the small intestine, binds to plasma proteins (99%) and is rapidly biotransformed in the liver into its deacetylated active metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB, **Figure 1**) (Ramis et al., 1991; McNeely and Goa, 1998; Cho et al., 2003) In healthy humans, the half-life of triflusal is *ca.* 0.5 h, whereas for HTB it is *ca.* 35 h (Gonzalez-Correa and De La Cruz, 2006). From a pharmacological point of view, it is interesting to note that HTB is itself highly active as a platelet anti-aggregant agent. Indeed, studies on the clinical profile of both drug and metabolite have shown no significant differences between them (Ramis et al., 1991).

Although their side effects are mainly gastrointestinal, it has been evidenced that both triflusal and HTB display ability to induce photoallergy in humans (Serrano et al., 1987; Lee et al., 1999, 2001; Nagore et al., 2000). This phenomenon involves a cell-mediated immune response, which is initiated by covalent binding of a light-activated photosensitizer (or a species derived therefrom) to a protein, a process known as haptenation (Ariza et al., 2011). Interestingly, although aspirin presents a similar chemical structure, it is not associated with photosensitivity disorders. Therefore, as mentioned above, the trifluoromethyl group at position 4 present in triflusal and HTB should be responsible for the photosensitizing properties of the molecule (Bosca et al., 2001; Caffieri et al., 2007; Montanaro et al., 2009).

Ubiquitin is a small (8.5 KDa) regulatory protein, with 76 amino acids, present in all eukaryotic cells, which was discovered in the early 1970s (Pickart and Eddins, 2004; Herrmann et al., 2007; Hochstrasser, 2009).

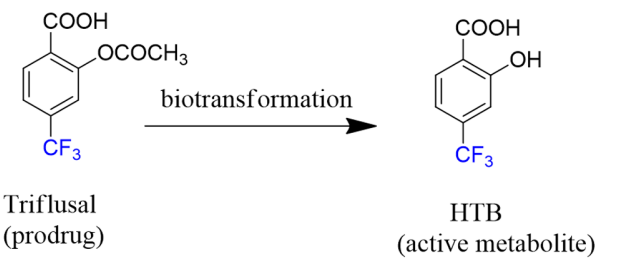


FIGURE 1 | Chemical structures of triflusal and its metabolite HTB.

Covalent ubiquitination is a major regulatory post-translational process, involving attachment of the ubiquitin to lysine residue/s on a substrate protein or on another ubiquitin molecule, leading to mono or polyubiquitination (Dikic et al., 2009; Suryadinata et al., 2014). Thus, monobiquitination can modify protein activity and localization by endocytosis, cell-cycle control or lysosomal targeting (Dikic et al., 2009), whereas polyubiquitination is implicated in DNA repair and immune signaling (Dikic et al., 2009; McIntyre and Woodgate, 2015). Besides, the discovery that ubiquitin chains target proteins to the proteasome, which degrades and recycles proteins, was honored with the Nobel Prize in chemistry in 2004.

Moreover, ubiquitin does not have a well-defined active site although it binds to the so-called ubiquitin binding sites, which are modular protein domains that non-covalently bind ubiquitin (Hicke et al., 2005; Hurley et al., 2006; Dikic et al., 2009) and reveal information about the functionality and the mechanism of intermolecular regulation.

In this context, small proteins like ubiquitin could be appropriate models for investigating covalent binding by means of MS/MS and peptidic fingerprint analysis (Jeram et al., 2009; Hong et al., 2015; Ramirez et al., 2015). In previous work, it was shown that HTB forms covalent photoadducts with isolated lysine and polylysine (Montanaro et al., 2009). Interestingly, ubiquitin contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys 63) that could be modified by a similar reaction.

With this background, the goal of the present work is to identify possible adduct formed between the triflusal metabolite and ubiquitin as model protein, upon sunlight irradiation, combining proteomic and photophysical (fluorescence and laser flash photolysis) techniques.

MATERIALS AND METHODS

Materials and Solvents

HTB was purchased from WAKO (Osaka, Japan) and was used without further purification; *n*-butylamine and bovine ubiquitin, whose sequence is identical to the human protein, were provided from Sigma–Aldrich (Steinheim, Germany). Phosphate buffered saline solution (PBS, pH = 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate amount of deionized water. Sephadex G-25 columns (PD-10) were acquired from Amersham Pharmacia Biotech (UK). Dichloromethane and ethyl acetate were from Scharlab (Sentmenat, Spain).

Photoaddition of HTB to Ubiquitin

Solutions containing HTB (5×10^{-5} M) and ubiquitin (5×10^{-5} M) in PBS were incubated 1 h in the dark. Samples were then irradiated for 1 h (18.9 J/cm^2) under the sunlight. For all photophysical studies, the photoadduct was separated from the protein using guanidine chloride and disposable Sephadex G-25 columns (PD-10) (Amersham Pharmacia Biotech, UK) equilibrated with PBS and the final product was further lyophilized at −55°C for 16 h. Control

included drug–protein mixture kept in the dark and ubiquitin without irradiation.

Synthesis of HTB-*n*-Butylamine Adduct

A mixture of HTB (150 mg, 0.73 mmol) and *n*-butylamine (720 μL, 7.3 mmol) dissolved in deaerated PBS solution (20 mL) was placed in quartz tubes and irradiated overnight by means of a multilamp photoreactor equipped with six lamps (Hitachi, F15T8/BL) with a maximal output at *ca.* 300 nm (Gaussian distribution). The crude product was dissolved in ethyl acetate, washed with 1 M HCl and brine, dried over $MgSO_4$ and concentrated under vacuum. The residue was washed with dichloromethane several times to get HTB-butylNH_2 as a white solid (94 mg, 63%). ^{1}H NMR (300 MHz, CD_3OD): δ 0.98 (t, J = 7.3 Hz, 3H), 1.38–1.45 (m, 2H), 1.56–1.66 (m, 2H), 3.32–3.40 (m, 2H), 7.29 (dd, J = 8.2 Hz and 1.6 Hz, 1H), 7.34 (d, J = 1.6 Hz, 1H), 7.93 (d, J = 8.2 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 14.2, 21.2, 32.5, 40.9, 116.4, 117.0, 118.5, 131.9, 142.4, 163. 0 169.1, 173.0. Exact mass: m/z found, 236.0922 calculated for $C_{12}H_{14}NO_4$ $(M\text{-}H)^+$ 236.0928.

Laser Flash Photolysis Experiments

Laser flash photolysis (LFP) experiments were carried out with a pulsed XeCl excimer laser (λ_{exc} = 308 nm, *ca.* 17 ns pulse width, <100 mJ per pulse). In general, samples received between 1 and 3 pulses for all the kinetic experiments. A pulsed Lo255 Oriel Xenon lamp was used as detecting light source. The observation wavelength was selected with a 77200 Oriel monochromator, and the signal amplified by an Oriel photomultiplier tube (PMT) system made up of a 77348 side-on tube, 70680 housing and a 70705 power supply. The signal was registered with a TDS-640A Tektronix oscilloscope and subsequently transferred to a personal computer. The absorbance of the solutions was adjusted at *ca.* 0.21 at the excitation wavelength. All transient spectra were recorded at room temperature using 10 mm × 10 mm quartz cells with 4 mL capacity and were bubbled for 15 min with N_2 before acquisition.

Fluorescence Measurements

Steady-state fluorescence experiments were carried out using a Photon Technology International (PTI, Germany) LPS-220B spectrofluorometer, equipped with a monochromator in the range of 200–700 nm. Time-resolved fluorescence measurements were performed with a Time Master fluorescence lifetime spectrometer TM 2/2003 from PTI, using a hydrogen/nitrogen flash lamp as the excitation source. The kinetic traces were fitted by monoexponential decay functions, using a deconvolution procedure to separate them from the lamp pulse profile. Emission measurements were performed in the region of 330–600 nm. The absorbance of the solutions was adjusted at ca. 0.08 at 308 nm. All measurements were performed at room temperature using 10 mm × 10 mm quartz cells of 4 mL capacity, under aerobic conditions.

Mass Spectrometry Analysis of HTB-Modified Ubiquitin by MALDI-TOF

Solutions containing HTB (5×10^{-5} M) and ubiquitin (5×10^{-6} M) in PBS were incubated 1 h in the dark, after which they were exposed to sunlight (doses of UVA light of 18.9 J/cm^2). Incubation mixtures were directly mixed with the matrix and applied to the MALDI-TOF analysis on an Autoflex III MALDI-TOF-TOF mass spectrometer (Bruker), operated in the positive mode as previously described (Renedo et al., 2007; Oeste et al., 2011).

Protein Digestion and LC-ESI-MS/MS Analysis

Solution containing HTB (5×10^{-5} M) and ubiquitin (5×10^{-6} M) in PBS was incubated 1 h in the dark and irradiated by sunlight. The samples were enzymatically digested into smaller peptides using trypsin. Subsequently, these peptides were analyzed using nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS). Briefly, 20 μg of sample were taken (according to Qubit quantitation) and the volume was set to 20 μL. Digestion was achieved with sequencing grade trypsin (Promega, trypsin: protein ratio 1:20 w/w) V = 64 μL, overnight 37°C. Digestion was stopped with 7 μL 10% TFA (trifluoroacetic acid). The final peptide mixture was at a concentration *ca* 0.5 μg/μL.

Next, 5 μL of sample were loaded onto a trap column (NanoLC Column, 3 μ C18 CL, 100 umx 15 cm; Nikkyo) and desalted with 0.1% TFA at 2 μL/min during 10 min. The peptides were then loaded onto an analytical column (LC Column, 3 μ C18 CL, 75 umx12 cm, Nikkyo) equilibrated in 5% acetonitrile 0.1% formic acid. Elution was carried out with a linear gradient of 5–40% B in A for 30 min (A: 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). The tripleTOF was operated in information-dependent acquisition

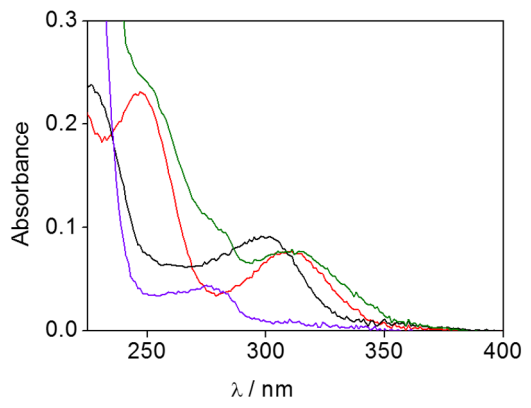


FIGURE 2 | UV-Vis spectra of phosphate buffer solutions of HTB (black), ubiquitin (violet), HTB-ubiquitin adduct (green) and HTB-ButylNH_2 adduct (red).

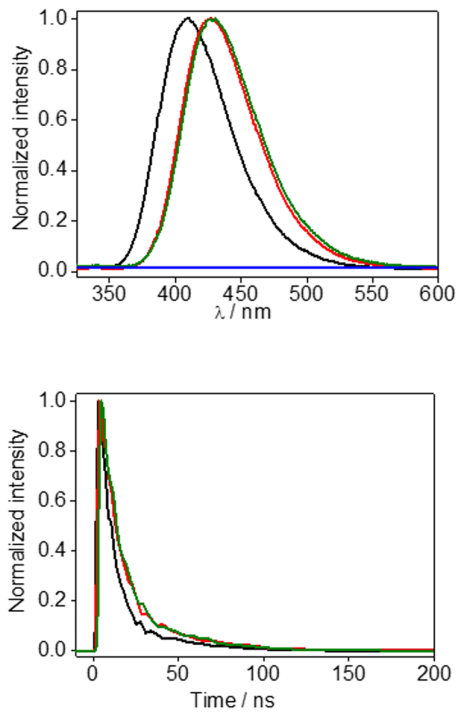


FIGURE 3 | (Top) Steady-state fluorescence (λ_{exc} = 308 nm) of phosphate buffer solution of HTB (black line), sunlight irradiated HTB-ubiquitin (1:1) after sephadex filtration (green line) or non-irradiated HTB-ubiquitin (1:1) after sephadex filtration (blue line), and HTB-butylNH$_2$ adduct (red line). **(Bottom)** Corresponding decays.

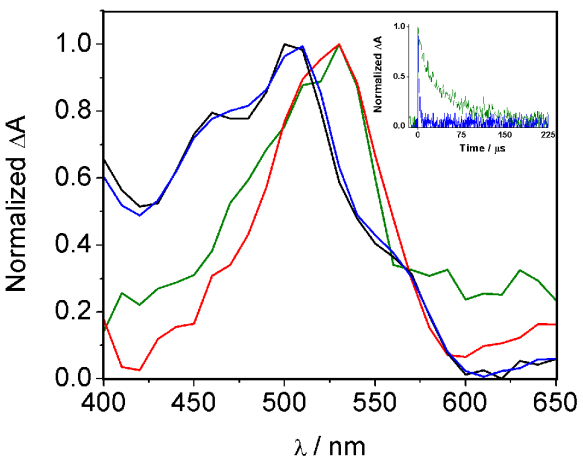


FIGURE 4 | Normalized transient absorption spectra of HTB (black line), HTB/ubiquitin 1:1 (blue line), HTB-ubiquitin (green line) and HTB-butylNH$_2$ (red line) adducts in phosphate buffer N_2-flushed solutions, 0.5 μs after the laser pulse. Inset: normalized decay traces of HTB /ubiquitin 1:1 (blue) and HTB-ubiquitin adduct (green).

mode, in which a 0.25-s TOF MS scan from 350 to 1250 m/z was performed, followed by 0.05-s product ion scans from 100 to 1500 m/z on the 10 most intense 2–5 charged ions.

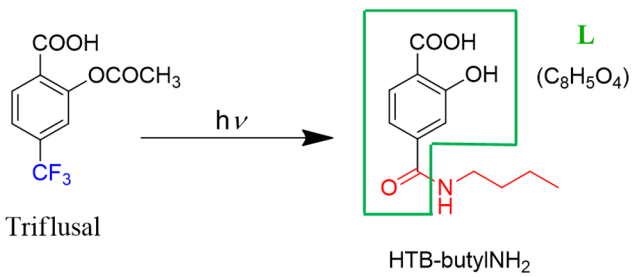
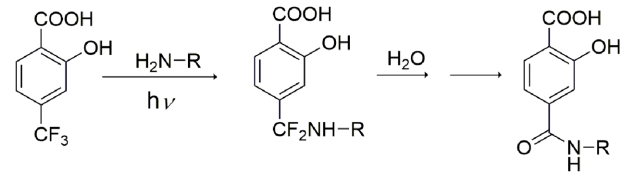


FIGURE 5 | Photoadduct HTB-butylNH$_2$ formation.



SCHEME 1 | Proposed mechanism of HTB-ubiquitin adduct formation.

ProteinPilot v4.5. (ABSciex) search engine default parameters were used to generate peak list directly from 5600 TripleTOF wiff files. The obtained mgf was used for identification with MASCOT (v 4.0, Matrix- Science). Database search was performed on Home Made (includes sequence of interest and the contaminants described in Expasy). Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 75 ppm in MS mode and 0.6 Da in MS/MS mode. Oxidation of Met and deamidation of Asn and Gln as variable modifications. HTB modification was set as variable for K, S, T.

RESULTS AND DISCUSSION

Sunlight irradiation of buffered aqueous solutions of HTB (5×10^{-5} M, **Figure 1**) and ubiquitin (1:1 molar ratio) was performed at noon in Valencia (Spain, July). Then, the protein material was separated from the free metabolite by gel-filtration chromatography (Sephadex). The high-molecular weight fraction was examined spectroscopically to reveal the presence of a covalently linked chromophore. As a control a 1:1 solution of HTB:ubiquitin was kept in the dark and filtered by Sephadex. This way the comparison between irradiated and non-irradiated samples would inform about the formation of a covalent adduct between the metabolite and the protein.

A first approach was based on UV-Vis spectrophotometry, so the absorption spectrum of the obtained proteinaceous fraction was registered together with those of ubiquitin and HTB for comparison. The protein alone in solution showed a band with a maximum at 270 nm and no significant absorption at $\lambda > 300$ nm (**Figure 2**, violet line); by contrast HTB displayed a UVB-band centered at 300 nm

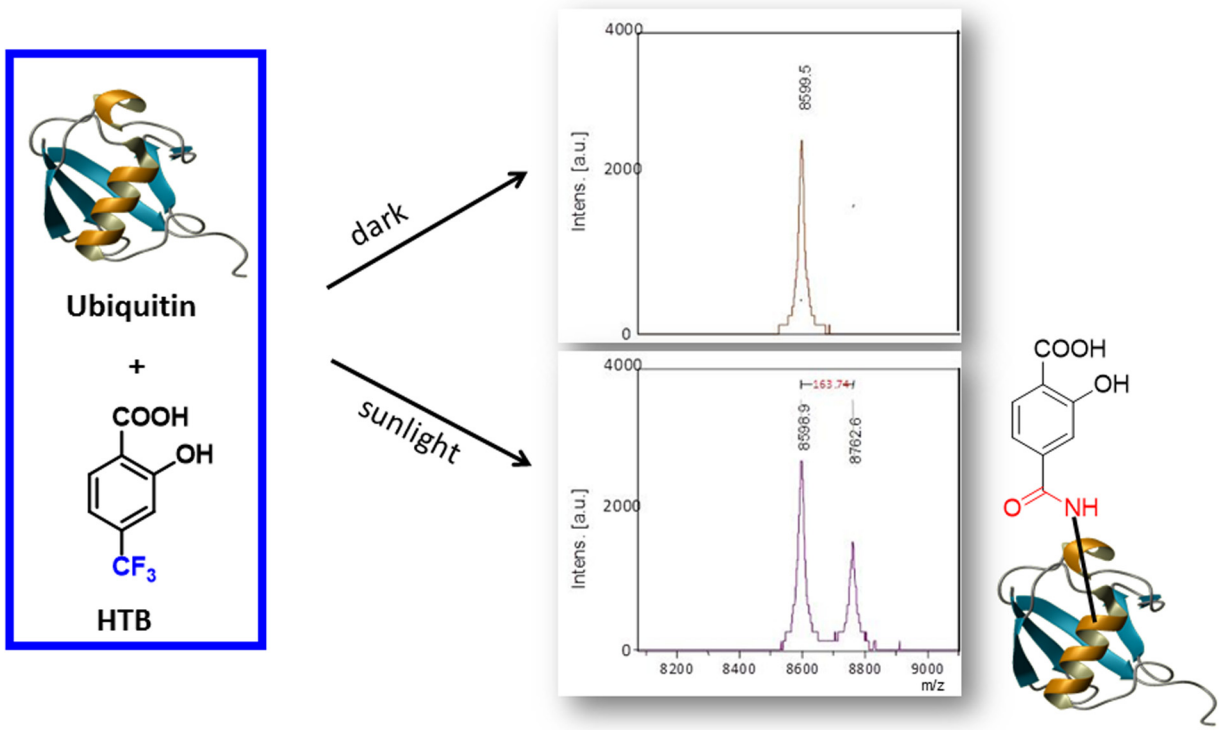


FIGURE 6 | MALDI-TOF spectra of HTB-ubiquitin (10:1) kept in the dark (Top) or solution after 30 min of sunlight irradiation (Bottom).

TABLE 1 | Identification of HTB-modified peptide by MS/MS spectrometry.

Peptide adduct ID	Observed precursor ion (m/z)	Charge (z)	Mr (exp)	Mr (calcd)	Sequence	Adduct site
1	715.7316	2	1428.7316	1428.7323	$_{1}$MQIFVK#TLTGK$_{11}$	K6
2	818.0825	3	2451.2257	2451.2268	$_{7}$TLTGK#TITLEVEPSDTIENVK$_{27}$	K11
3	717.6962	3	2150.0668	2150.0630	$_{12}$TITLEVEPSDTIENVK#AK$_{29}$	K27
4	844.3987	2	1686.7828	1686.7849	$_{30}$IQDK#EGIPPDQQR$_{42}$	K33
5	755.8790	2	1509.7434	1509.7463	$_{43}$LIFAGK#QLEDGR$_{54}$	K48
6	765.3953	3	2293.1641	2293.1590	$_{55}$TLSDYNIQK#ESTLHLVLR$_{72}$	K63

(**Figure 2**, black line). Interestingly, the irradiated and filtered HTB:ubiquitin solution clearly exhibited an absorption peaking at 310 nm (**Figure 2**, green line), whereas no UVB-band was observed for the non-irradiated and filtered sample (see Supplementary Material). This result points toward the formation of a covalent adduct between the protein and the metabolite.

To get further insight, steady-state and time-resolved fluorescence experiments were conducted. At the excitation wavelength of 308 nm, HTB emission was detected at *ca.* 405 nm in agreement with previous reports, (Bosca et al., 2001; Montanaro et al., 2009) whereas a red shifted fluorescence spectrum, with λ_{em} at *ca.* 420 nm, was obtained for the irradiated and filtered HTB-ubiquitin sample (**Figure 3** top, green line). No emission was detected for the control solution kept in the dark (**Figure 3** top, blue line). Moreover, time-resolved experiments revealed different lifetimes for the HTB and HTB-ubiquitin samples, τ being of *ca.* 9.5 and 13.5 ns, respectively (**Figure 3** bottom, black and green lines).

Additional spectroscopic studies of the covalent adduct between HTB and ubiquitin were performed by nanosecond LFP using a 308 nm XeCl excimer laser for excitation. The transient spectra registered for a nitrogen-flushed solution of HTB alone or irradiated and filtered HTB-ubiquitin in phosphate buffer solutions are shown in **Figure 4**. The HTB-ubiquitin adduct exhibited a transient absorption centered at 520 nm, a wavelength close to that observed for the HTB triplet excited state at λ_{TT} of *ca.* 500 nm (Bosca et al., 2001; Montanaro et al., 2009).

Thus, detection of a spectroscopic response for the high molecular weight fraction of the irradiated HTB:ubiquitin solution evidenced the formation of a covalent photoadduct.

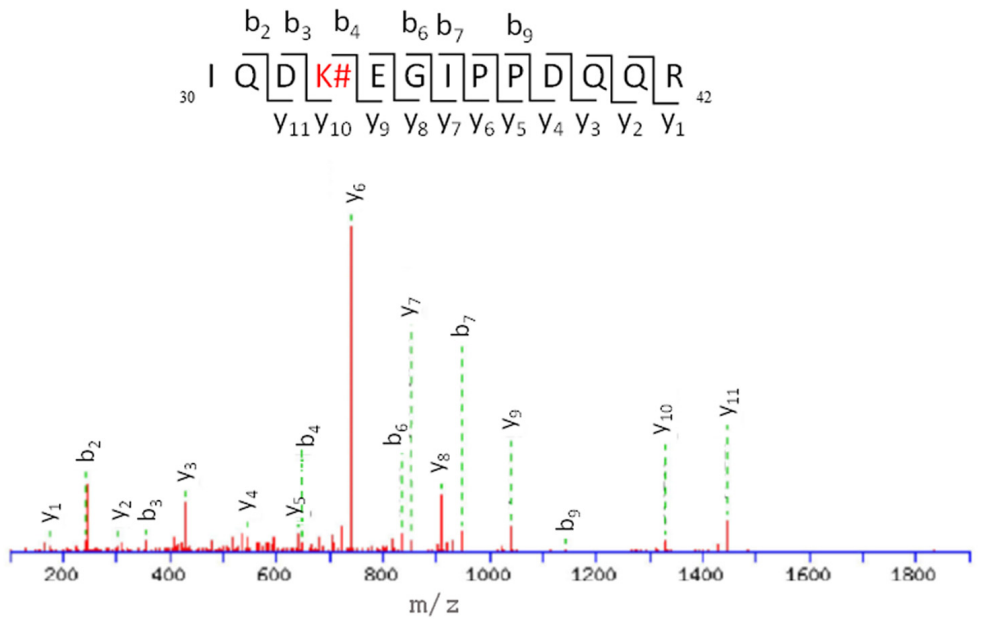


FIGURE 7 | ESI-MS/MS spectra and fragmentation pathway of the HTB-modified peptide 4.

Furthermore, the spectral similarities between the free metabolite and this proteinaceous fraction indicated the presence of a HTB-like chromophore in the modified ubiquitin biomolecule.

Thus, based on the previous studies that have established photoaddition of HTB at the ε-amino group of lysine, (Montanaro et al., 2009) the model compound (HTB-butylNH$_2$, **Figure 5**) was synthesized by UVB-irradiation of a phosphate buffer solution of *n*-butylamine and HTB. Photoadduct HTB-butylNH$_2$ was obtained as main product in 63% yield and fully characterized by NMR and HRMS (see Supplementary Material). The spectroscopic characterization of this model compound in phosphate buffer revealed a strong analogy between its photobehavior and that of HTB-ubiquitin photoadduct. Indeed, it displayed the same UVB absorption maximum (**Figure 2**, red line), a coincident fluorescence emission and lifetime (**Figure 3**), as well as an identical transient absorption spectrum (**Figure 4**).

Altogether, these results support formation of a HTB-ubiquitin adduct through photoaddition at the ε-amino group of lysine (**Scheme 1**). To obtain more precise structural information, the photoreactivity of HTB with the whole ubiquitin biomolecule was addressed by MALDI-TOF and HPLC-nanoESI analysis. Comparison between the MALDI-TOF spectra of sunlight irradiated solutions of ubiquitin alone (5×10^{-5} M, *m/z* 8599) and HTB-ubiquitin (10:1) mixture revealed the appearance of a new peak at *m/z* 8763 that corresponds to an increment of 164 amu (**Figure 6**), compatible with L(-H). Similar results were obtained for 1:1 ratio. Next, incubation mixtures were filtered to remove excess HTB and trypsin digestion followed by HPLC-MS/MS was performed in order to investigate the modified peptide sequence and to undertake a detailed characterization of the HTB-ubiquitin adduct. Full scan, as well as fragmentation, data files were analyzed by means of the Mascot® database search engine (Matrix Science, Boston, MA, USA) and by entering variable modifications that take into account the main nucleophilic sites able to react with the trifluoromethyl group of HTB, i.e., Lys, Thr and Ser, (Caffieri et al., 2007; Montanaro et al., 2009). Results confirmed identification of six HTB-ubiquitin derived peptide adducts: $_{1}$MQIFVKTLTGK$_{11}$, $_{7}$TLTGKTITLEVEPSDTIENVK$_{27}$, $_{12}$TITLEVEPSDTIENVKAK$_{29}$, $_{30}$IQDKEGIPPDQQR$_{42}$, $_{43}$LIFAGKQLEDGR$_{54}$, $_{55}$TLSDYNIQKESTLHLVLR$_{72}$ (all peptides have one missed cleavage at Lys), their related data are summarized in **Table 1**.

Thus, the modification site of each peptide was assessed by tandem mass experiments on the trypsin digests. The MS/MS fragmentation was achieved by selecting the precursor ions given in **Table 1**. The peptide sequence well agreed with the *y* and *b* ion series (see Supplementary Material). Here, the case of fragment 4, namely $_{30}$IQDKEGIPPDQQR$_{42}$, is discussed as an example (**Figure 7**); further information on the others fragments is given in the Supplementary Material. The MS/MS fragment ions showed an unmodified *y* ion series from y_1 to y_9, whereas an increment of *m/z* 292 L(-H)-Lys(-H_2O) was detected between y_9 to y_{10}. Accordingly, the *b* ion series suffered the same increase from b_3 to b_4. Thus, the modified amino acid is the Lys 33. Examination of the other five tryptic peptides confirms that Lys is the main site for the adduct formation with modifications detected at K_6, K_{11}, K_{27}, K_{48}, and K_{63}. It should be mentioned that no diagnostic *y* and *b* fragment ions were found for Lys 27 and 29; however, detection of peptide 3 points toward formation

of an adduct at site 27, which represents a missed cleavage due to the bulky L substituent that likely hinders trypsin ability to cleave at this site.

CONCLUSION

Irradiation of HTB, the active metabolite of triflusal, in the presence of ubiquitin under sunlight gives rise to covalent photobinding. Photophysical and proteomic analysis demonstrate that although the main product found is a monoadduct, the reaction take places at all the ε-amino groups of the lysine residues of the protein and involves replacement of the trifluoromethyl moiety with a new amide function. Concentrations of both drug and protein used in the present work are compatible with those present in blood plasma. Therefore, it is expected that immunologic reactions occur in patients under triflusal therapy in combination with sunlight exposure. This process can in principle occur with other trifluoroaromatic compounds and may be responsible for the appearance of undesired photoallergic side effects.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00277

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Pharmacogenomics of Prostaglandin and Leukotriene Receptors

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Individual genetic background together with environmental effects are thought to be behind many human complex diseases. A number of genetic variants, mainly single nucleotide polymorphisms (SNPs), have been shown to be associated with various pathological and inflammatory conditions, representing potential therapeutic targets. Prostaglandins (PTGs) and leukotrienes (LTs) are eicosanoids derived from arachidonic acid and related polyunsaturated fatty acids that participate in both normal homeostasis and inflammatory conditions. These bioactive lipid mediators are synthesized through two major multistep enzymatic pathways: PTGs by cyclooxygenase and LTs by 5-lipoxygenase. The main physiological effects of PTGs include vasodilation and vascular leakage (PTGE2); mast cell maturation, eosinophil recruitment, and allergic responses (PTGD2); vascular and respiratory smooth muscle contraction (PTGF2), and inhibition of platelet aggregation (PTGI2). LTB4 is mainly involved in neutrophil recruitment, vascular leakage, and epithelial barrier function, whereas cysteinyl LTs (CysLTs) (LTC4, LTD4, and LTE4) induce bronchoconstriction and neutrophil extravasation, and also participate in vascular leakage. PTGs and LTs exert their biological functions by binding to cognate receptors, which belong to the seven transmembrane, G protein-coupled receptor superfamily. SNPs in genes encoding these receptors may influence their functionality and have a role in disease susceptibility and drug treatment response. In this review we summarize SNPs in PTGs and LTs receptors and their relevance in human diseases. We also provide information on gene expression. Finally, we speculate on future directions for this topic.

Keywords: prostaglandins, leukotrienes, eicosanoid receptors polymorphisms, inflammation, NSAID-hypersensitivity

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INTRODUCTION

Arachidonic acid is released from cell membrane phospholipids by phospholipase A2 in response to different stimuli, and then oxidized through cyclooxygenase (COX)-1 or 5-lipoxygenase (5LO) to produce prostaglandins (PTGs) and leukotrienes (LTs), respectively (Capra et al., 2015). Although these bioactive lipid mediators, collectively named eicosanoids, are associated with homeostasis they also play a key role in inflammation.

The COX-1 pathway firstly leads to the formation of prostanoids with the PTGH2 endoperoxide intermediate, that is metabolized to PTGD2, PTGE2, PTGF2a, PTGI2, and thromboxane (TX)A2 by specific synthases. The main physiological effects of PTGs include vasodilation and vascular leakage (PTGE2); mast cell maturation, eosinophil recruitment and allergic responses (PTGD2); vascular and respiratory smooth muscle contraction (PTGF2), and inhibition of platelet aggregation (PTGI2) (Claar et al., 2015).

LTs can be grouped into the chemoattractant LTB4 and the cysteinyl LTs (CysLTs): LTC4, LTD4, and LTE4. LTB4 is mainly involved in neutrophil recruitment, vascular leakage, and epithelial barrier function, whereas CysLTs induce bronchoconstriction and neutrophil extravasation, and also participate in vascular leakage (Singh et al., 2013).

PTGs and LTs exert their biological functions by binding to cognate receptors belonging to the seven transmembrane G protein-coupled receptor superfamily (GPCRs): PTGFR and PTGIR for PTGF and PTGI2, respectively; PTGDR and CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) for PTGD; PTGER1 to PTGER4 for PTGE; and TXA2R for TXA2 (Thompson et al., 2014). LTB receptors are BLTR1 and BLTR2, and CysLTR1 and CysLTR2 for CysLTs.

In addition to the environment, human complex diseases are also affected by individual factors including genetic background. In fact, a number of genetic variants, mainly single nucleotide polymorphisms (SNPs), have been consistently associated with different pathological and inflammatory conditions, representing potential targets for therapy. SNPs in genes encoding PTG and LT receptors may influence their functionality and have a role in disease susceptibility and treatment response. In this review we summarize current knowledge regarding SNPs in PTGs and LTs receptors and their association with human inflammatory conditions (**Table 1**), with a special focus on hypersensitivity to non-steroidal anti-inflammatory drugs (NSAIDs), the most common type of drug hypersensitivity.

PTG, TBXA2, AND LT RECEPTOR POLYMORPHISMS IN INFLAMMATORY CONDITIONS

PTGFR, PTGIR, PTGDR, and *CRTH2*

Current data concerning *PTGFR* genetic variation supports its relationship with variability in treatment response to latanoprost, a commonly prescribed antiglaucomatous drug, although the mechanisms underlying this association are unknown (Gao et al., 2015; Ussa et al., 2015).

PTGI2 (prostacyclin) plays a key role in the cardiovascular system through the specific inhibition of platelet aggregation and its vasodilatory effects on smooth muscle (Dorris and Peebles, 2012). The polymorphism A984C in *PTGIR* has been associated with platelet aggregation and may play a role in cerebral infarction (Shimizu et al., 2013). Two synonymous variants in this gene (V53V and S328S) have also been associated with enhanced platelet activation in patients with deep vein thrombosis (Patrignani et al., 2008).

An important genomic region on chromosome 14 that includes *PTGDR* has been linked to asthma and atopy (1997; Mansur et al., 1999; Hakonarson et al., 2002). There are conflicting results concerning the association of promoter variants influencing the transcription of this gene with these phenotypes. For example, some studies have failed to associate two variants in *PTGDR* (−441C>T and −197TC>T) with asthma using family-based or case-control analyses in Latinos and African American asthmatics (Tsai et al., 2006). In addition, particular *PTGDR* haplotypes could not be associated with Chinese children with asthma and atopy (Leung et al., 2009), or with two Australian populations of asthmatics (Jamrozik et al., 2011), with similar data having been previously reported in a distinct Chinese population (Li et al., 2007). However, −441C>T, −197TC>T, −549T>C, and various haplotypes have been associated with asthma in two ethnically different populations (Oguma et al., 2004). Moreover, the −197T >C and −613C >T polymorphisms have been significantly associated with allergic asthma, and allergy to pollen and mites, respectively (Isidoro-García et al., 2011). The same authors also found that the *PTGDR* diplotype CCCT/CCCC (−613CC, −549CC, −441CC, and −197TC), with a potential influence on gene transcription, was more frequent in patients with nasal polyposis, with or without asthma, and in patients suffering from the aspirin triad (Benito Pescador et al., 2012). In addition to these SNPs in *PTGDR*, the promoter polymorphism −731A>G and the intronic variant 6651C>T were found to be associated with asthma, atopic asthma, and bronchial hyperreactivity in families from UK, with similar results for 6651C>T in a Danish study (Zhu et al., 2007). The intronic variants rs17831675 and rs17831682 in *PTGDR* have also been associated with asthma susceptibility (Ungvári et al., 2012). These variants, as well as rs17125273, also appear to have some relevance with respect to IgE levels in asthmatics (Ungvári et al., 2012).

Different studies have shown certain polymorphisms in *CRTH2* (rs11571288, rs545659, and rs634681) to be associated with asthma and allergic sensitization in different populations (Huang et al., 2004; Cameron et al., 2009; Wang et al., 2009). A functional study showed that in addition to being linked with asthma, the 1544G–1651G haplotype in the *CRTH2* 3′-UTR increased mRNA stability, supporting its role as a strong candidate gene for asthma (Huang et al., 2004). In another study the rs533116 variant was associated with a higher proportion of CRTh2 cells during Th2 differentiation as well as increased IL-4 and IL-13 expression levels after agonist stimulation (Campos Alberto et al., 2012). The minor allele of *CRTH2* 1431G>C has been also associated with levels of specific IgE to food allergens in a population of German children (Cameron et al., 2009).

PTGE Receptors

Considering the great variety of biological functions carried out by PTGE it is not surprising that SNPs in its receptors appear to have a role in different pathologies. For example, the rs17197 polymorphism in the 3′-UTR of *PTGER2* has been associated with essential hypertension in men (Sato et al., 2007), and the intronic rs2268062 variant in *PTGER3* with hypertension in a separate study (Sõber et al., 2009). The latter may be explained

TABLE 1 | Main prostaglandin and leukotriene polymorphisms associated with different human inflammatory conditions.

Gene	Polymorphism	Condition	Population	References
Prostaglandin I2 receptor (PTGIR)	A984C	Cerebral infarction	Japanese	Shimizu et al., 2013
	V53V, S328S	Deep vein thrombosis	Italian	Patrignani et al., 2008
	1915T>C	NERD	Korean	Thompson et al., 2007
Prostaglandin D2 receptor (PTGDR)	−441C>T, −549T>C, and −197T>C haplotypes	Asthma	American	Oguma et al., 2004
	−613C>T	Allergy to pollen and mites	Spanish	Isidoro-García et al., 2011
	−197T>C	Allergic asthma	Spanish	Isidoro-García et al., 2011
	−613C>T, −549T>C, −441C>T, and −197T>C diplotype	Nasal polyposis with and without asthma, and aspirin triad	Spanish	Benito Pescador et al., 2012
	−731A>G, 6651C>T	Asthma, atopic asthma, and bronchial hyperreactivity	British and Danish	Zhu et al., 2007
	rs8004654	NIUA	Spanish	Cornejo-Garcia et al., 2012
Prostaglandin D2 receptor 2 (CRTH2)	1544G>C, 1651A>G	Asthma and IL13 levels	Chinese	Wang et al., 2009
	1538A>G	Food allergy sensitization	German	Cameron et al., 2009
	−466T>C	NERD	Korean, and Japanese	Palikhe et al., 2010; Kohyama et al., 2012
	−466T>C	Antihistamine requirements in chronic urticaria	Korean	Palikhe et al., 2009
Prostaglandin E receptor 1 (PTGER1)	1544G>C, 1651A>G	Asthma	African American, Chinese	Huang et al., 2004
	rs3810253, rs3810255	NERD	Spanish	Ayuso et al., 2015
Prostaglandin E receptor 2 (PTGER2)	rs17197	Essential hypertension	Japanese	Sato et al., 2007
	−616C>G, −166G>A	NERD	Korean	Kim et al., 2007b
	rs1254598	NERD	Spanish	Ayuso et al., 2015
Prostaglandin E receptor 3 (PTGER3)	rs977214	Protective effect in preterm birth	Norwegian	Ryckman et al., 2010
	1388T>C	Increased asthma risk	Korean	Park et al., 2007
Prostaglandin E receptor 4 (PTGER4)	rs7720838	Crohn disease	American and German, German	Glas et al., 2012; Prager et al., 2014
	rs4434423	Primary graft dysfunction	Multicentered cohort including different ethnicities	Diamond et al., 2014
	rs10440635	Ankylosing spondylitis	Han Chinese	Chai et al., 2013
	−1254G>A	NERD	Korean	Kim et al., 2007b
	rs13186505, rs4133101	NIUA	Spanish	Cornejo-Garcia et al., 2012
	rs4495224	NERD	Spanish	Ayuso et al., 2015
	−1254G>A	NECD	Korean	Palikhe et al., 2012
Thromboxane A2 receptor (TBXA2R)	rs768963	Cerebral infarction	Han Chinese	Shao et al., 2015
	rs768963	Cerebral infarction, large artery atherosclerosis, and small artery occlusion subtype.	Chinese	Zhao et al., 2013
	795T>C	NERD	Korean, Japanese	Kim et al., 2007b; Kohyama et al., 2012

(Continued)

TABLE 1 | Continued

Gene	Polymorphism	Condition	Population	References
Cysteinyl leukotriene receptor 1 (CYSLTR1)	G300S	Atopy and asthma	Tristan da Cunha	Thompson et al., 2007
	634C>T	NECD	Korean	Kim et al., 2007a
	rs320995	Asthma	Spanish	Arriba-Mendez et al., 2006
	927T>C	Atopy severity	British	Hao et al., 2006
	rs320995	NIUA	Spanish	Cornejo-Garcia et al., 2012
	−634C>T, −475A>C, −336A>G	NERD	Korean	Kim et al., 2006
	−634C>T	NERD	Korean	Kim et al., 2007c
Cysteinyl leukotriene receptor 2 (CYSLTR2)	M201V	Atopy	Tristan da Cunha	Thompson et al., 2003
	601A>G	Asthma	Danish and American	Pillai et al., 2004
	−1220A>C	Asthma	Japanese	Fukai et al., 2004
	rs320995	NERD	Spanish	Ayuso et al., 2015
	−819T>G, 2078C>T, 2534A>G	NERD	Korean	Park et al., 2005

NECD, non-steroidal anti-inflammatory drugs-exacerbated cutaneous disease; NERD, non-steroidal anti-inflammatory drugs-exacerbated respiratory disease; NIUA; non-steroidal anti-inflammatory drugs-induced urticaria/angioedema.

by the role of this gene in neurotransmitter release modulation in central and peripheral neurons, and in the inhibition of sodium and water reabsorption in kidney (Breyer and Breyer, 2000). A protective effect in preterm birth has been suggested for the minor allele of rs977214 in *PTGER3* (Ryckman et al., 2010).

Concerning asthma, a Bayesian analysis found an association for several SNPs in *PTGER2* (rs12587410, rs17197, rs1254600, rs708498) (Ungvári et al., 2012). A Korean study showed the promoter variant rs207579 (−616C>G) in *PTGER2* to be associated with increased asthma risk whereas two variants in the 3′-UTR of *PTGER3* (rs959 and rs34745168) were associated with a diminished risk (Park et al., 2007).

Environmental and genetic determinants affecting *PTGER4* expression might be thought to be crucial in a plethora of diseases, giving the central role of its ligand, PTGE2, in immunomodulation and control of inflammation (Soontrapa et al., 2011; Tang et al., 2012; Konya et al., 2013). For example, it has been shown for Crohn's disease that disease-associated alleles in a gene-desert region in 5p13.1 correlate with expression levels of *PTGER4*, the closest gene to this region (Libioulle et al., 2007). Through a meta-analysis of genome wide association studies and replication studies, rs11742570 in *PTGER4* was recently found to be associated with inflammatory bowel disease (Jostins et al., 2012).

Two polymorphisms in *PTGER4* (rs4495224 and rs7720838) have been associated with susceptibility to Crohn's disease in three independent cohorts (Glas et al., 2012). Using *in silico* analysis the authors predicted these SNPs to be crucial components of binding sites for the transcription factors NF-κB and XBP1 with higher binding scores for carriers of the risk alleles, which could explain how these SNPs contribute to increased *PTGER4* expression (Glas et al., 2012). Notably, the association of *PTGER4* rs7720838 with Crohn's disease has recently been replicated in other populations (Prager et al., 2014).

Although the molecular link between genetic variation and altered regulatory T cell function in primary graft dysfunction has not been uncovered, a recent study showed the major allele of the rs4434423 variant in *PTGER4* to be associated with their differential suppressive functions in this pathology (Diamond et al., 2014).

Finally, another SNP in *PTGER4* (rs10440635) has been associated with ankylosing spondylitis severity in a Han Chinese population (Chai et al., 2013).

TXA2R

TXA2R plays a central role in atherosclerosis and thrombosis (Capra et al., 2014). It has been recently demonstrated that the promoter polymorphism rs768963 is more frequent among Han Chinese who have suffered from cerebral infarction (Shao et al., 2015). Cells containing two exonic variants (C795T and T924C) showed an increased ligand binding-induced intracellular calcium influx and fibrinogen-integrin conjugation, suggesting that *TXA2R* SNPs may influence platelet function and the risk of developing cerebral ischemia (Shao et al., 2015). In another study the C allele of rs768963 was associated with large artery atherosclerosis and small artery occlusion subtypes (Zhao et al., 2013).

The variants V80E and A160T in *TBXA2R* showed opposing roles on platelet activity, inhibiting and promoting platelet activation respectively (Gleim et al., 2013). The *TBXA2R* 924TT genotype has been associated with residual platelet activity after off-pump artery bypass grafting (Wang et al., 2013), and with resistance to aspirin (Gao et al., 2011). This genotype also showed less effectiveness in platelet aggregation (Fujiwara et al., 2007). Homozygosity for the minor allele of the C795T, C924T or the G1686A *TBXA2R* SNPs was associated with a decreased expression of CD62P in agonist stimulated platelets (Fontana

et al., 2006). Finally, the *TBXA2R* SNP rs13306046 was associated with decreased blood pressure (Nossent et al., 2011).

A recent meta-analysis has shown an association between the *TBXA2R* 924C>T polymorphism and asthma (Pan et al., 2016). Asthmatic children with combinations of the *TBXA2R* 795T>C and 924T>C risk alleles had higher total IgE levels, total eosinophil counts, and lower FEV1 than those carrying the common alleles (Kim et al., 2008). The minor allele frequency of *TBXA2R* 795T>C was significantly higher in NSAIDs-exacerbated respiratory disease (NERD) patients compared with aspirin-tolerant asthmatics and homozygous patients for the C allele had a greater percent fall of FEV1 compared with carriers of the CT or TT genotypes (Kim et al., 2005). A significant association was also observed between the *TBXA2R* T924C polymorphism and FEV1 in children with atopic asthma (Leung et al., 2002; Hong et al., 2005).

Two haplotypes involving four intronic SNPs (rs2238631, rs2238632, rs2238633, and rs2238634) influenced transcriptional activity and were associated with asthma-related phenotypes (Takeuchi et al., 2013). These SNPs are in linkage disequilibrium with the exonic SNPs rs11318632 and rs4523. The first one was associated with atopic asthma (Shin et al., 2003), and the latter with adult asthma (Unoki et al., 2000) and atopic asthma in children (Leung et al., 2002). A suggestive association between rs8113232 in *TBXA2R* and rhinitis in asthmatic children has been also reported (Kavalar et al., 2012). Finally, the *TBXA2R* 4684T allele is associated with lower *TBXA2R* expression, which may contribute to the development of NSAIDs-exacerbated cutaneous disease (NECD) (Palikhe et al., 2011).

LTB4R1 and *LTB4R2*

Gene structure analysis of lung samples has revealed that both *LTB4R1* and *LTB4R2* show splice variation in the 5′-UTR and multiple promoter regions, although they did not find susceptibility markers for asthma development (Tulah et al., 2012). A previous study failed to associate polymorphisms spanning the *LTBR* locus with baseline lung function in smokers (Tulah et al., 2011).

Increased cytotoxicity and migration of NK cells appears to be linked to LTB4 (Wang et al., 2015). However, there is a differential role for its two receptors. Using a selective receptor antagonist, LTB4R1 was shown to be involved in both LTB4-induced migration and cytotoxicity, whereas LTB4R2 was involved exclusively in NK cell migration, but only in response to higher concentrations of LTB4. The expression of these two receptors increased after activation of NK cells with IL-2 and IL-15, and these changes in expression were reflected in enhanced NK cell responses to LTB4 (Wang et al., 2015).

CYSLTR1 and *CYSLTR2*

The functional variant G300S in *CYSLTR1* was associated with atopy in the Tristan da Cunha population (Thompson et al., 2007) as the M201V SNP in *CYSLTR2* was previously (Thompson et al., 2003). The G300S association has been replicated in another study showing that LTD4-induced phosphorylation of *Erk* is higher in transfected cells (Yaddaden et al., 2016).

Significant differences in genotype frequencies of the *CYSLTR1* promoter polymorphism −634C>T have been associated with clinical requirement for leukotriene receptor antagonists (Kim et al., 2007a). In addition, patients with the variant genotype showed higher expression levels of *CYSLTR1* than those with the common genotype (Kim et al., 2007a).

The rs320995 variant in *CYSLTR1* has been associated with asthma in two independent studies in Spain (Arriba-Mendez et al., 2006; Sanz et al., 2006). Although a UK study involving 341 families could not find this association, it was linked to atopy severity in females (Hao et al., 2006).

A significant association between the coding polymorphism 601A>G in *CYSLTR2* and asthma was observed in a family-based study of asthmatics from Denmark and USA, with the G allele appearing to be less frequently transmitted to asthmatics (Pillai et al., 2004). Using a calcium mobilization assay the authors demonstrated that carriers of the G allele, LTD4 was approximately five-fold less potent compared to wild type, suggesting a potential mechanism for resistance to asthma (Pillai et al., 2004).

A transmission disequilibrium test of 137 Japanese asthmatic families showed that the −1220A>C SNP in *CYSLTR2* was associated with asthma development and with atopic asthma in Japan (Fukai et al., 2004).

The mRNA and protein expression of *CYSLT1R* and *CYSLT2R* was found to be significantly increased in polyp tissues from eosinophilic chronic rhinosinusitis patients, and this increase was shown to be related to LTC4 and LTD4 levels (Wu et al., 2016). *CYSLTR2* is expressed in the lungs of asthmatics and its activation appears to contribute to antigen-induced bronchoconstriction in certain asthma populations (Sekioka et al., 2015).

PTG, TBXA2, AND LT RECEPTOR POLYMORPHISMS IN NSAID HYPERSENSITIVITY

NSAIDs, commonly used in the treatment of pain and some inflammatory diseases, are the main triggers of drug hypersensitivity reactions (Conaghan, 2012). The most frequent type of reaction caused by these drugs is cross-intolerance, in which COX-1 inhibition leads to CysLTs release, resulting in clinical symptoms in susceptible individuals (Doña et al., 2011, 2012, 2014). Three main phenotypes of cross-intolerance are currently recognized: NERD (previously known as aspirin-induced asthma and ASA triad), in patients with rhinitis and/or asthma with/without nasal polyposis; NECD (previously known as aspirin-intolerant chronic urticaria), in patients with underlying chronic idiopathic urticaria; and NSAIDs-induced urticaria/angioedema (NIUA) in otherwise healthy individuals (Kowalski et al., 2013).

Recent years have witnessed increased interest in the potential role of SNPs in these pathologies. We will now focus on those variants related to PTG and LT receptors.

PTGFR, PTGIR, PTGDR, and *CRTH2*

Most genetic association studies have not further investigated the functional effects of associated variants, for example, the 1915T>C variant in *PTGIR* that has been associated with NERD

in Korea, however the mechanisms by which it leads to the pathology are unclear (Kim et al., 2007b).

Although the *PTGDR* −549T>C (rs8004654) SNP could not been shown to be associated with NERD in a Korean population (Kim et al., 2007b), we recently found an association between NIUA, the most common type of drug hypersensitivity reactions, and this polymorphism in two independent Spanish populations (Cornejo-Garcia et al., 2012). In terms of potential mechanism, it has been shown that the C allele (risk for NIUA in our study) increases GATA-2/GATA-3 transcription factor binding, modifying promoter activity and *PTGDR* gene expression (Oguma et al., 2004). Interestingly, given that an association could not be found in the Korean study mentioned above, this variant has also been associated with NERD in Spain (Ayuso et al., 2015).

In patients with NERD the *CRTH2* −466T>C polymorphism increases both serum and cellular eotaxin-2 production, and may lead to eosinophilic infiltration in these patients (Palikhe et al., 2010). Another study also showed that the frequency of the homozygous genotype for the less frequent allele of *CRTH2* −466T>C was higher in NERD patients compared to aspirin-tolerant asthmatics (Kohyama et al., 2012). Interestingly, homozygous TT chronic urticaria patients required higher doses of antihistamines (Palikhe et al., 2009).

PTGE Receptors

Two promoter variants (rs3810253 and rs3810255) in *PTGER1* have recently been found to be associated with NERD, although their functional consequences need to be elucidated (Ayuso et al., 2015).

We have found that the rs1254598 polymorphism in *PTGER2* was marginally associated with NERD patients when compared with NSAID-tolerant asthmatics (Ayuso et al., 2015). In addition, two promoter variants in *PTGER2* (−616C>G and −166G>A) have been associated with NERD in a Korean population (Kim et al., 2007b).

It has been shown that in patients with NECD the GG genotype at −1254G>A in *PTGER4* is more frequent than in healthy individuals (Palikhe et al., 2012). In addition, the G allele leads to lower *in vitro* promoter activity with decreased *PTGER4* expression (Palikhe et al., 2012). This promoter polymorphism (−1254A>G) has also been reported to be associated with NERD in Koreans (Kim et al., 2007b). However, recent research failed to demonstrate an association between NIUA and two promoter variants in *PTGER4* (rs13186505 and rs4133101) in a Spanish population (Cornejo-Garcia et al., 2012). In addition, no statistically significant association was found for another variant in *PTGER4* (rs4495224) in NERD patients (Ayuso et al., 2015).

TXA2R

The frequencies of the CC/CT genotype of *TBXA2R* 795T>C were higher than those of the TT genotype in NERD patients compared to ASA-tolerant asthmatics (Kohyama et al., 2012). *TBXA2R* −4684C>T and 795T>C variants have also been associated with NERD (Kim et al., 2007b).

CYSLTR1 and *CYSLTR2*

Male NERD patients showed higher frequencies of the minor alleles of −634C>T, −475A>C, −336A>G polymorphisms in *CYSLTR1* than male controls (Kim et al., 2006). In addition, the haplotype [T-C-G] was associated with increased disease risk for NERD and also affected gene expression, measured by significantly enhanced luciferase activity (Kim et al., 2006). The −634C>T SNP was also most frequent in NERD compared to NECD patients and the variant genotype showed significantly higher promoter activity than the common genotype (Kim et al., 2007c).

The *CYSLTR1* variant rs320995 was associated with NIUA in two independent Spanish populations (Cornejo-Garcia et al., 2012). It has also been associated with NERD in Spain (Yaddaden et al., 2016), but not with NERD in Korea (Choi et al., 2004).

Finally, NERD patients carrying the minor alleles for 819T>G, 2078C>T or 2534A>G in *CYLSTR2* exhibited a more pronounced fall in FEV1 after aspirin provocation than those who carried the common allele (Park et al., 2005).

FUTURE DIRECTIONS

Important international efforts such as HapMap and the 1000 Genomes projects, as well as advances in *in silico* analysis have led to substantial advances in our knowledge of the human genome and its relationship with disease. This will almost certainly increase in the near future through the adoption of next-generation sequencing approaches for the discovery of associated variants, and the integration of the different omics technologies, such as transcriptomics, metabolomics, and lipidomics, which will help us to uncover the underlying mechanisms behind complex human diseases.

Focusing on GPCRs, a number of public databases integrating genome data and *in silico* predictions are currently available and have been recently reviewed (Thompson et al., 2014). Four recommended computational tools for predicting the functional effects of SNPs on protein structure and function are PolyPhen (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.jcvi.org), SNAP (https://www.broadinstitute.org/mpg/snap), and Mutation Taster (http://www.mutationtaster.org).

Mediators from the arachidonic acid pathway play a key role in inflammation as well as in the pathogenesis of a number of diseases. The lack of agreement in genetic association studies is likely related to population differences, including ethnic genetic background, sample size, and differences in phenotype definitions. In addition, most genetic studies have not addressed rare variants that are thought to be involved in complex diseases together with environmental factors. In spite of these limitations, as technologies improve and international networks and collaborations continue to flourish, we expect our knowledge on the genetic basis of inflammation and other conditions to become more refined, potentially leading to the discovery of markers and treatment options for a range of diseases.

AUTHOR CONTRIBUTIONS

JC, JP, RJ, NS, and NB revised the literature available on this topic and wrote the first manuscript draft. JC, EG, JA, and EV revised

the manuscript. JC and JP are responsible of the final English version.

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FCERI and Histamine Metabolism Gene Variability in Selective Responders to NSAIDS

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The high-affinity IgE receptor (Fcε RI) is a heterotetramer of three subunits: Fcε RIα, Fcε RIβ, and Fcε RIγ (αβγ2) encoded by three genes designated as *FCER1A*, *FCER1B* (*MS4A2),* and *FCER1G*, respectively. Recent evidence points to FCERI gene variability as a relevant factor in the risk of developing allergic diseases. Because Fcε RI plays a key role in the events downstream of the triggering factors in immunological response, we hypothesized that FCERI gene variants might be related with the risk of, or with the clinical response to, selective (IgE mediated) non-steroidal anti-inflammatory (NSAID) hypersensitivity. From a cohort of 314 patients suffering from selective hypersensitivity to metamizole, ibuprofen, diclofenac, paracetamol, acetylsalicylic acid (ASA), propifenazone, naproxen, ketoprofen, dexketoprofen, etofenamate, aceclofenac, etoricoxib, dexibuprofen, indomethacin, oxyphenylbutazone, or piroxicam, and 585 unrelated healthy controls that tolerated these NSAIDs, we analyzed the putative effects of the FCERI SNPs *FCER1A* rs2494262, rs2427837, and rs2251746; *FCER1B* rs1441586, rs569108, and rs512555; *FCER1G* rs11587213, rs2070901, and rs11421. Furthermore, in order to identify additional genetic markers which might be associated with the risk of developing selective NSAID hypersensitivity, or which may modify the putative association of FCERI gene variations with risk, we analyzed polymorphisms known to affect histamine synthesis or metabolism, such as rs17740607, rs2073440, rs1801105, rs2052129, rs10156191, rs1049742, and rs1049793 in the *HDC*, *HNMT,* and *DAO* genes. No major genetic associations with risk or with clinical presentation, and no gene-gene interactions, or gene-phenotype interactions (including age, gender, IgE concentration, antecedents of atopy, culprit drug, or clinical presentation) were identified in patients. However, logistic regression analyses indicated that the presence of antecedents of atopy and the DAO SNP rs2052129 (GG) were strongly related ($P < 0.001$ and $P = 0.005$, respectively) with selective hypersensitivity to ibuprofen. With regard to patients with selective hypersensitivity to ASA, men were more prone to develop such a reaction than women ($P = 0.011$), and the detrimental *DAO* SNP rs10156191 in homozygosity increased the risk of developing such hypersensitivity ($P = 0.039$).

Keywords: Fcε RI, histamine, non-steroidal anti-inflammatory drugs (NSAIDS), hypersensitivity drug reactions, biomarkers

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INTRODUCTION

Type B drug-induced hypersensitivity reactions (DHR) occur only in susceptible individuals with a frequency of 5–10% of all adverse drug reactions (Khan and Solensky, 2010). In general, these reactions are severe and occasionally may be life-threatening. In recent years evidence has accumulated to support the notion that DHR may be caused by various mechanisms and is the result of a complex multifactorial and multigenic process (Pirmohamed, 2006). Regarding culprit drugs, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequent causes of DHR together with antibiotics (Cornejo-Garcia et al., 2009; Doña et al., 2011, 2012; Kowalski et al., 2011). Hypersensitivity reactions to a single NSAID (selective reactions) are the result of an immunological mechanism, which is either IgE-mediated in acute reactions or T cell-mediated in delayed reactions. Clinical manifestations include cutaneous reactions, respiratory reactions and anaphylaxis, which may appear with different onset times after drug intake. Histamine release from mast cells after IgE receptor activation plays a relevant role in allergic inflammation and in the development of clinical symptoms (Mita et al., 2001; Kowalski et al., 2011).

A priori, genetic variations affecting both components (expression and/or function) of this signaling pathway, including high affinity IgE receptor and histamine metabolizing enzymes, could modify the risk of developing hypersensitivity to NSAIDs, and/or the presentation of clinical manifestations. In fact, recent studies have demonstrated an association between several allergic diseases including drug hypersensitivity and polymorphisms in these genes (García-Martín et al., 2006, 2007a; Kennedy et al., 2008; Gervasini et al., 2010; Maintz et al., 2011).

The high-affinity IgE receptor (Fcε RI) is a heterotetramer of three subunits: Fcε RIα, Fcε RIβ, and Fcε RIγ ($\alpha\beta\gamma_2$) encoded by three genes designated as *FCER1A*, *FCER1B* (*MS4A2*), and *FCER1G*, respectively. The genes coding for all FcεRI subunits are polymorphic and some of these polymorphisms have been associated with several atopic diseases (MacGlashan et al., 1998, 1999; Saini et al., 1999; Weidinger et al., 2008; Zhang et al., 2010; Li et al., 2014). The *FCER1A* gene is located on chromosome 1q23 (1:159259504-159278014; 1000 Genomes, 2015), and it encodes a protein with two extracellular IgE-like domains with ligand-binding sites (Potaczek and Kabesch, 2012). The *FCER1B* gene is located on chromosome 11q12-13 (11:59855734–59863444; 1000 Genomes, 2015), and it encodes a 244-amino acid protein with a non-canonic intracellular ITAM domain. Functionally, it is a signal-augmenting subunit. The *FCER1G* gene is located on chromosome 1q23 (1:161185024–161190489; 1000 Genomes, 2015) and encodes an 86-amino acid protein. Functionally, it is a signal-transducing subunit and plays an essential role in the induction of mast cell degranulation and survival (Manikandan et al., 2012).

With regard to histamine homeostasis, histamine biosynthesis is catalyzed by the enzyme L-histidine decarboxylase (HDC, E.C. 4.1.1.22). Two enzymes participate in the degradation of histamine: Histamine N-methyltransferase (HNMT, E.C. 2.1.1.8) and diamine oxidase (DAO; E.C. 1.4.3.6; García-Martín et al., 2009). There is high individual variability in histamine metabolism which is, at least in part, genetically determined, although other factors, such as gender (García-Martín et al., 2007b) play a major role in the variability of histamine metabolism. An association between polymorphisms of histamine metabolizing enzymes and the clinical presentation of allergic diseases has been demonstrated (García-Martín et al., 2006, 2007a; Kennedy et al., 2008; Gervasini et al., 2010; Maintz et al., 2011).

The *HDC* gene is located in chromosome 15q21-q22 and spans around 24 kilobases. The *HNMT* gene is located in chromosome 2q22.1. The human *DAO* gene spans ∼10 kb and is located in chromosome 7q34-q36. A promoter SNP and three common non-synonymous SNPs have been identified in Caucasian individuals, and the functional effects of these SNPs in enzyme activity have been studied in detail (Ayuso et al., 2007; García-Martín et al., 2007b).

In order to investigate the role of genetic factors in the risk of developing selective NSAID hypersensitivity, both isolated and combined, given that the clinical outcome depends on the interplay of IgE response and the consequent release of mediators, in this study we analyzed functional polymorphisms in high affinity IgE receptors and genes related to histamine metabolism in a large group of well-phenotyped patients suffering from single-NSAID-induced hypersensitivity reactions. Our aim was to elucidate the putative association of these gene polymorphisms with several clinical phenotype parameters, including gender, age, antecedents of atopy, culprit drug, IgE levels and clinical presentation, and to analyze putative gene-gene interactions. The final goal was to identify biomarkers which alone or combined with pharmacogenomics or metabolic biomarkers (Agúndez et al., 2009a,b, 2015; Cornejo-García et al., 2012; Martinez et al., 2014; García-Martín et al., 2015a; Ariza et al., 2016) might be useful in prevention, diagnosis and/or proper management of these patients.

PATIENTS AND METHODS

Study Population

We studied a cohort of 899 individuals, consisting of 314 unrelated Spanish patients with single-NSAID-induced hypersensitivity reactions and 585 unrelated healthy controls. Written consent for participation was obtained for all participants. All the patients who were invited to participate in the study agreed to do so. Of the patients, 145 were recruited from the Allergy service, Hospital Infanta Leonor (Madrid, Spain), 100 were recruited from the Allergy service, Regional Hospital (Málaga, Spain) and 69 were recruited from the Allergy Department, Infanta Cristina Hospital (Badajoz, Spain). Diagnosis was carried out as previously described (Doña et al., 2011). After confirming good tolerance to a full therapeutic dose of ASA, incremental doses of the culprit drug were given until the therapeutic dose for the analgesic/NSAIDs implicated in the study was achieved. In the case of selective responses to ASA,

confirmation of the selective response was made by challenge with indomethacin as reported elsewhere (Doña et al., 2011).

Participants' characteristics are summarized in **Table 1**. The NSAIDs responsible for the reaction are summarized in **Table 2**. Clinical presentation distributed according to gender and culprit drug are shown in **Table 3**.

The healthy controls were recruited from staff and medical students of the Hospitals and the Universities participating in the study, and were ethnically matched with patients (all were unrelated Spanish individuals). All control subjects tolerated the NSAIDs most frequently involved in selective hypersensitivity, as shown in **Table 2**. Specifically, all control individuals previously received metamizole, ibuprofen, diclofenac, paracetamol, and ASA, and experienced no hypersensitivity or other ADRs. Individuals with the above-mentioned characteristics were asked to participate, and 97% of them agreed. A medical history was obtained and an examination was performed for each participant to exclude pre-existing disorders. Individuals with familial (up to second-degree relatives) or personal antecedents of allergic, atopic, or autoimmune diseases were excluded from the control group to avoid confounders. The protocol for this study was in accordance with the Declaration of Helsinki and its subsequent revisions and was approved by the respective Ethics Committees of the participating Hospitals.

TABLE 1 | Characteristics of the study group.

	Patients ($n = 314$)	Healthy subjects ($n = 585$)
Women, *n* (%)	205 (65.3)	356 (60.8)
Age [*SD*; range]	45.4 [16.1; 5–82]	22.3 [4.8; 20–57]
Antecedents of atopy, *n* (%)	66 (21.0)	0
Antecedents of urticaria, *n* (%)	4 (1.3)	0
Interval <1 h; *n* (%), Single NSAID-induced urticaria/angioedema, and anaphylaxis	232 (73.9)	n.a.
Interval >24 h; *n* (%), Single NSAID-induced delayed reaction	13 (4.1)	n.a.
Interval unknown, *n* (%)	69 (22.0)	n.a.

TABLE 2 | Culprit drug for selective NSAID-induced hypersensitivity.

Culprit drug	Total No. (%)	PATIENTS DISTRIBUTED BY GENDER	
		Men (No. %)	Women (No. %)
Metamizole	108 (34.4)	32 (29.3)	76 (37.1)
Ibuprofen	94 (29.9)	35 (32.1)	59 (28.8)
Diclofenac	35 (11.1)	15 (13.8)	20 (9.8)
Paracetamol	20 (6.4)	7 (6.4)	13 (6.3)
Acetyl salicylic acid	19 (6.1)	8 (7.3)	11 (5.4)
Propifenazone	12 (3.8)	4 (3.7)	8 (3.9)
Naproxen	9 (2.9)	3 (2.8)	6 (2.9)
Ketoprofen	3 (1.0)	0	3 (1.5)
Dexketoprofen	3 (1.0)	2 (1.8)	1 (0.5)
Etofenamate	3 (1.0)	1 (0.9)	2 (1.0)
Aceclofenac	1 (0.3)	1 (0.9)	0
Etoricoxib	1 (0.3)	0	1 (0.5)
Dexibuprofen	1 (0.3)	0	1 (0.5)
Indomethacin	1 (0.3)	0	1 (0.5)
Oxyphenbutazone	1 (0.3)	0	1 (0.5)
Piroxicam	1 (0.3)	0	1 (0.5)
Unknown	2 (0.6)	1 (0.9)	1 (0.5)
Total	314 (100)	109 (100)	205 (100)

Genotype Analysis

Genomic DNA was obtained from peripheral leukocytes and purified in accordance with standard procedures. The SNPs analyzed were selected according to allele frequencies (over 0.01) in the study population, and either functional or clinical relevance, in line with published evidence (Preuss et al., 1998; García-Martín et al., 2006, 2007a, 2009; Ayuso et al., 2007; Maintz et al., 2011; Amo et al., 2016), and the public 1000 genomes database release of 17 Nov. 2015. Genotyping was performed by TaqMan assays (Life Technologies, Alcobendas, Madrid, Spain). Details of the TaqMan probes and the allele frequencies in Caucasian individuals are summarized in **Table 4**. We studied nine *FCER1* SNPs, as well as SNPs corresponding to genes involved in histamine synthesis (*HDC*) or metabolism *(HNMT and DAO*). All these histamine-related SNPs have demonstrated functional and/or clinical implications (García-Martín et al., 2006, 2007a,b, 2009, 2015b; Ayuso et al., 2007; Gervasini et al., 2010; Agúndez et al., 2012).

Detection was carried out by means of real-time PCR (qPCR) in an Eppendorf realplex thermocycler using fluorescent probes. The amplification conditions were as follows: After a denaturation time of 10 min at 96°C, 45 cycles of 92°C 15 s 60°C 90 s were carried out and fluorescence was measured at the end of each cycle and at endpoint. All samples were determined in triplicate and genotypes were assigned both by means of gene identification software (RealPlex 2.0, Eppendorf), and by analysis of the reference cycle number for each fluorescence curve, calculated using the CalQPlex algorithm (Eppendorf).

Statistical Analyses

Statistical power for each SNP was evaluated with a genetic model to analyze the frequency for the minor allele with an odds ratio (OR) value = 1.5 ($\alpha = 0.05$) based on the allele frequencies observed in the control group. **Table 4** shows the statistical power for each SNP analyzed. For most SNPs the statistical power was very high. In some cases, because of the low minor allele frequency observed, the statistical power was not sufficient to detect an OR = 1.5 but was sufficient to detect an OR = 2.0 with a bilateral power of more than 80% (**Table 4**), with the single exception of the HDC SNP rs2073440 T/G, whose power was sufficient to detect an OR = 2.1.

SNPStats software (Solé et al., 2006) was used to calculate allele and genotype frequencies, to analyze the Hardy-Weinberg equilibrium, and to determine linkage disequilibrium statistics and haplotype frequency estimation. Multiple comparison adjustment was done by using the False Discover Rate (FDR) correction (http://www.sdmproject.com/utilities/?show=

TABLE 3 | Clinical presentation of selective NSAID-induced hypersensitivity.

Gender	Urticaria + Angioedema (No. %)	Anaphylaxis (No. %)	Exanthema (No. %)	Mixed pattern (No. %)	Respiratory (No. %)	Toxic hepatitis (No. %)	Unknown (No. %)	Total (No. %)
PATIENTS DISTRIBUTED BY CLINICAL PRESENTATION								
Men	57 (52.3)	40 (36.7)	9 (8.3)	2 (1.8)	0	0	1 (0.9)	109 (100)
Women	113 (55.1)	68 (33.2)	10 (4.9)	7 (3.4)	5 (2.4)	1 (0.5)	1 (0.5)	205 (100)
Culprit drug	**Urticaria + Angioedema (No. %)**	**Anaphylaxis (No. %)**	**Exanthema (No. %)**	**Mixed pattern (No. %)**	**Respiratory (No. %)**	**Toxic hepatitis (No. %)**	**Unknown (No. %)**	**Total (No. %)**
Metamizole	45 (41.7)	53 (49.1)	7 (6.5)	3 (2.8)	0	0	0	108 (100)
Ibuprofen	70 (74.5)	18 (19.1)	2 (2.1)	3 (3.2)	1 (1.1)	0	0	94 (100)
Diclofenac	13 (37.1)	20 (57.1)	2 (5.7)	0	0	0	0	35 (100)
Paracetamol	12 (60.0)	5 (25.0)	1 (5.0)	0	2 (10.0)	0	0	20 (100)
Acetyl Salicylic Acid	13 (68.4)	1 (5.3)	2 (10.5)	0	2 (10.5)	0	1 (5.3)	19 (100)
Propifenazone	6 (50.0)	4 (33.3)	1 (8.3)	1 (8.3)	0	0	0	12 (100)
Naproxen	4 (44.4)	2 (22.2)	1 (11.1)	2 (22.2)	0	0	0	9 (100)
Ketoprofen	2 (66.7)	0	0	0	0	1 (33.3)	0	3 (100)
Dexketoprofen	1 (33.3)	2 (66.6)	0	0	0	0	0	3 (100)
Etofenamate	2 (66.7)	0	1 (33.3)	0	0	0	0	3 (100)
Aceclofenac	0	1 (100)	0	0	0	0	0	1 (100)
Etoricoxib	1 (100)	0	0	0	0	0	0	1 (100)
Dexibuprofen	0	1 (100)	0	0	0	0	0	1 (100)
Indomethacin	0	0	1 (100)	0	0	0	0	1 (100)
Oxyphenbutazone	0	0	0	0	0	0	1 (100)	1 (100)
Piroxicam	1 (100)	0	0	0	0	0	0	1 (100)
Unknown	0	1 (50)	1 (50)	0	0	0	0	2 (100)
Total	170	108	19	9	5	1	2	314

TABLE 4 | SNPs analyzed in this study.

Gene	Chromosomal location	dbSNP	Consequence	Assay ID	MAF (1000 genomes, European individuals)	Statistical power (two tailed, OR = 1.5, α =0.05)* (%)
FCER1A	1:159253672	rs2494262 A/C	Upstream gene	C____494924_20	0.44 C	98
FCER1A	1:159258545	rs2427837 G/A	Upstream gene	C__16233438_20	0.30 A	96
FCER1A	1:159272060	rs2251746 T/C	Intronic	Custom-designed	0.30 C	96
FCER1B	11:59856028	rs1441586 T/C	5 prime UTR	C___1842226_10	0.46 C	98
FCER1B	11:59863104	rs569108 A/G	Missense 237 E/G	C____900116_10	0.04 G	36 (a)
FCER1B	11:59863253	rs512555 C/T	3 prime UTR	C___7513065_10	0.04 T	37 (b)
FCER1G	1:161184875	rs11587213 A/G	Upstream gene	C__27848237_10	0.18 G	88
FCER1G	1:161185058	rs2070901 G/T	Non-coding transcript exon	C__15867981_20	0.27 T	97
FCER1G	1:161188936	rs11421 T/C	3 prime UTR	C___1841966_1_	0.15 C	91
HNMT	2:138759649	rs11558538 C/T	Missense 105 T/I	C__11650812_20	0.10 T	73 (c)
DAO	7:150548972	rs2052129 G/T	Upstream gene	C__11630976_1	0.25 T	97
DAO	7:150553605	rs10156191 C/T	Missense 16 T/M	C__25593951_10	0.27 T	96
DAO	7:150554553	rs1049742 C/T	Missense 332 S/F	C___7599782_20	0.08 T	58 (d)
DAO	7:150557665	rs1049793 C/G	Missense 645 H/D	C___7599774_10	0.27 G	97
HDC	15:50534514	rs2073440 T/G	Missense 644 E/D	C__15950871_20	0.02 G	31 (e)
HDC	15:50555544	rs17740607 G/A	Missense 31 T/M	C__25624415_20	0.10 A	69 (f)

**The statistical power (two tailed, OR = 2.0, α = 0.05) is as follows: (a) 82%; (b) 83%; (c) 100%; (d) 97%; (e) 75%; (f) 99%. MAF, Minor allele frequency.*

FDR). Analyses of association with a response variable (culprit drugs and reactions) based on logistic regression were performed using SPSS 21.0 for Windows. For these analyses, we also determined the odds ratio and the corresponding 95% confidence intervals. The Hosmer-Lemeshow goodness of fit test for logistic regression was used. For the IgE response variable a multiple linear regression model was calculated. The results were considered as statistically significant when the *p*-value was less than 0.05.

RESULTS

The percentage of women was slightly higher in the cases, as compared to the control individuals (**Table 1**), although no statistically significant gender differences between cases and controls were present (Chi-square $P = 0.191$). Conversely, age was lower in the controls than in the cases. However, age is not a key factor in this study, as all the control individuals were tolerant to NSAIDs and the odds are extremely low that they may eventually develop selective NSAID-induced hypersensitivity.

The most common culprit drugs for selective NSAID-induced hypersensitivity were metamizole, ibuprofen, diclofenac, paracetamol, and ASA (**Table 2**). Although the frequency for metamizole-induced hypersensitivity was higher in women than in men, OR = 1.42 and 95% confidence interval (CI) 0.84–2.41; $P = 0.171$, the frequency difference was not statistically significant, and neither were the differences for gender-related frequencies for the rest of the NSAIDs included in **Table 2**. These frequencies correspond to those previously described by our group (Doña et al., 2011; Blanca-López et al., 2016a,b).

The most frequent clinical presentation was urticaria + angioedema, followed by anaphylaxis, exanthema and mixed pattern. No gender-related differences in clinical presentation were observed (**Table 3**). Clinical presentation, however, was strongly related with the culprit drug: The clinical presentation urticaria + angioedema was particularly frequent when the culprit drug was ibuprofen (OR = 3.50, 95% CI = 1.99–6.19); $P < 0.001$. In contrast, when the causative drug was metamizole or diclofenac, the most common clinical presentation was anaphylaxis (OR = 2.65, 95% CI = 1.58–4.44; $P < 0.001$) and (OR = 2.89, 95% CI = 1.34–6.28, $P = 0.003$), respectively. When the culprit drug was paracetamol or ASA the most frequent presentation was urticaria + angioedema, although for these two drugs the association of the drug with clinical presentation was not statistically significant. These phenotypic features correspond to those previously reported for selective NSAID hypersensitivity patients (Cornejo-Garcia et al., 2009; Doña et al., 2011; García-Martín et al., 2015a).

FCER1 genotyping results are summarized in **Table 5**. We checked the codominant, dominant, recessive, overdominant and additive models and the best fit was obtained with the recessive model. *FCER1* SNPs did not show statistically significant differences when patients and controls were compared in any of the genetic models analyzed. The genotyping results related to histamine synthesis and metabolism genes are summarized in **Table 6**. Once again, the best fit for histamine metabolism genes was obtained with the recessive model. Statistically significant differences on comparing cases and control subjects were identified for the *DAO* SNP rs10156191, which caused decreased enzyme activity (Ayuso et al., 2007). The observed difference was related to the frequency of homozygous individuals for the minor allele, which obtained a marginal significance that was not observed when the allele frequency (instead of the genotypes) was analyzed. When correction for multiple comparisons was carried out by using FDR, the P value for the recessive model for the DAO rs10156191 SNP was not significant (corrected $P = 0.294$), whereas the *P*-value for carriers of the minor allele for the HDC SNP rs2073440 remained significant (corrected *P*-value = 0.021).

Table 7 shows the statistically significant interaction of the genotypes studied and gender. The association of the *FCER1A* SNP rs2427837 with the risk of developing NSAID-induced hypersensitivity showed a positive interaction with gender, the association being stronger in women (**Table 7**). A statistically significant, genotype-gender interaction was observed for two other *FCER1* genotypes (rs2251746 in women and rs11587213 in men). When FDR correction for multiple comparisons (both genders and all SNPs) was made, the corrected *P*-values remained

TABLE 5 | *FCER1* SNPs analyzed in this study.

Gene	Chromosomal location	dbSNP	Cases non-mutated/ heterozygous/ homozygous	Cases MAF	Control non-mutated/ heterozygous/ homozygous	Control MAF	Comparison values (recessive model; OR, 95% CI)	Comparison values (carrier of the minor allele; OR, 95% CI)
FCER1A	1:159253672	rs2494262	83/146/73	0.483	136/260/107	0.471	1.12 (0.75–1.67); $P = 0.589$	1.05 (0.86–1.29); $P = 0.633$
FCER1A	1:159258545	rs2427837	181/101/17	0.226	297/212/38	0.263	0.73 (0.40–1.34); $P = 0.312$	0.82 (0.65–1.03); $P = 0.089$
FCER1A	1:159272060	rs2251746	185/107/14	0.221	300/221/34	0.260	1.22 (0.82–1.81); $P = 0.337$	1.10 (0.90–1.34); $P = 0.350$
FCER1B	11:59856028	rs1441586	82/148/71	0.482	160/275/114	0.458	1.22 (0.82–1.81); $P = 0.337$	1.10 (0.90–1.34); $P = 0.350$
FCER1B	11:59863104	rs569108	274/21/1	0.039	517/39/0	0.035	5.66 (0.23–139.3; $P = 0.170$	1.11 (0.66–1.88); $P = 0.690$
FCER1B	11:59863253	rs512555	290/22/1	0.038	530/39/0	0.034	5.48 (0.22–134.9); $P = 0.177$	1.12 (0.67–1.89); $P = 0.660$
FCER1G	1:161184875	rs11587213	219/79/15	0.174	408/150/16	0.159	1.75 (0.85–3.60); $P = 0.126$	1.12 (0.86–1.45); $P = 0.400$
FCER1G	1:161185058	rs2070901	164/124/25	0.278	285/231/52	0.294	0.84 (0.50–1.40); $P = 0.493$	0.92 (0.74–1.14); $P = 0.453$
FCER1G	1:161188936	rs11421	217/85/10	0.168	390/157/24	0.180	0.75 (0.35–1.60); $P = 0.452$	0.93 (0.71–1.20); $P = 0.553$

Three hundred and fourteen cases and 585 control individuals were included in the study. The sum of genotypes do not correspond to all cases and controls because of DNA shortage.

TABLE 6 | SNPs related with histamine synthesis and degradation analyzed in this study.

Gene	Chromosomal location	dbSNP	Cases non-mutated/ heterozygous/ homozygous	Cases MAF	Control non-mutated/ heterozygous/ homozygous	Control MAF	Comparison values (recessive model; OR, 95% CI)	Comparison values (carrier of the minor allele; OR, 95% CI)
HNMT	2:138002079	rs11558538	260/51/2	0.088	466/95/7	0.096	0.51 (0.11–2.48); $P = 0.398$	0.91 (0.65–1.28); $P = 0.580$
DAO	7:150851884	rs2052129	173/118/14	0.239	322/204/30	0.237	0.87 (0.45–1.68); $P = 0.676$	1.02 (0.80–1.27); $P = 0.928$
DAO	7:150856517	rs10156191	178/122/13	0.236	304/214/43	0.267	0.52 (0.27–0.99); $P = 0.042$	0.85 (0.68–1.06); $P = 0.160$
DAO	7:150857465	rs1049742	277/34/1	0.058	483/68/2	0.065	0.87 (0.08–9.66); $P = 0.911$	0.88 (0.58–1.33); $P = 0.540$
DAO	7:150860577	rs1049793	160/123/28	0.288	292/220/51	0.290	1.00 (0.61–1.65); $P = 0.994$	1.01 (0.81–1.25); $P = 0.940$
HDC	15:50242317	rs2073440	289/16/1	0.029	354/25/0	0.033	3.67 (0.15–90.52); $P = 0.269$	0.44 (0.26–0.77); $P = 0.003$
HDC	15:50263347	rs17740607	255/53/3	0.095	346/68/8	0.100	0.51 (0.13–1.94); $P = 0.313$	0.95 (0.67–1.35); $P = 0.770$

Three hundred and fourteen cases and 585 control individuals were included in the study. The sum of genotypes do not correspond to all cases and controls because of DNA shortage.

TABLE 7 | Statistically significant gender-related risk associations.

Gene	Chromosomal location	dbSNP	Gender	Cases MAF	Control MAF	Comparison values (recessive model; OR, 95% CI)	Comparison values (carrier of the minor allele; OR, 95% CI)
FCER1A	1:159258545	rs2427837	Women	0.198	0.279	0.64 (0.28–1.46); $P = 0.280$	0.64 (0.46–0.89); $P = 0.007$
FCER1A	1:159272060	rs2251746	Women	0.196	0.271	0.68 (0.28–1.63); $P = 0.380$	0.65 (0.47–0.91); $P = 0.010$
FCER1G	1:161184875	rs11587213	Men	0.229	0.129	2.42 (0.74–7.93); $P = 0.135$	2.01 (1.26–3.19); $P = 0.003$

significant: $P = 0.045$ for the genetic associations with the SNPs rs2427837 and rs2251746 and $P = 0.035$ for the SNP rs11587213.

Logistic regression analyses were carried out with separate models for each clinical presentation, as shown in **Table 3**, by comparing between cases all genotypes, gender, age, IgE concentration, and antecedents of atopy. In addition, we analyzed putative associations with response, stratifying patients into two groups: Single NSAID-induced urticarial/angioedema or anaphylaxis (SNIUAA) and single NSAID-induced delayed reactions (SNIDR). No significant associations were identified. In addition, logistic regression analyses were carried out with separate models for each culprit drug (only drugs with 19 or more cases were included, as shown in **Table 2**), by comparing between cases all genotypes, gender, age, IgE concentration, and antecedents of atopy.

For ibuprofen, the Hosmer–Lemeshow (HL) goodness of fit test was equal to 0.80 and revealed that age [$P = 0.032$; OR (95% CI) = 0.91 (0.83–0.99)], the presence of atopy antecedents [$P < 0.001$; OR = 19.61 (4.13–90.90)] and the DAO SNP rs2052129 (GG); [$P = 0.005$; OR = 13.25 (2.14–81.84)] were related to the risk of developing hypersensitivity. With regard to metamizole, although linear regression analysis suggested association with the absence of atopy ($P = 0.008$; OR = 5.80, 95% CI = 1.58–21.28), the HL goodness of fit test was equal to 0.001, and therefore the significance of these findings is limited. For ASA, we identified significant associations with the SNPs rs10156191 (TT) [$P = 0.035$, OR = 44.59; 95% CI = 1.22–1630.56] and with gender (for men, $P = 0.011$, OR = 25.64; 95% CI = 2.13–333.33) with an HL goodness of fit test equal to 0.957. No significant associations were observed with paracetamol or diclofenac. IgE levels did not show any association with phenomic or genomic markers. We did not identify any additional significant associations, although we cannot rule out association with other culprit drugs because the subgroup sizes were not sufficiently large to reach statistical significance.

DISCUSSION

NSAID-induced hypersensitivity type B adverse reactions are mediated by immunological and non-immunological mechanisms. Two major clinical phenotypes have been described: Selective NSAID hypersensitivity, which is drug-specific and an IgE-mediated mechanism, and cross-intolerance in which chemically non-related NSAIDs induce the reaction (Kowalski et al., 2013). Because selective hypersensitivity is an IgE-mediated mechanism, we analyzed genetic variations at the high-affinity IgE receptor, which has been shown to be related with allergic disorders (MacGlashan et al., 1998, 1999; Saini et al., 1999; Weidinger et al., 2008; Zhang et al., 2010; Li et al., 2014; Amo et al., 2016). It is to be noted that, despite the large body of published evidence supporting association of *FCER1* SNPs with allergic diseases, this is the first study to analyze the putative role of *FCER1* SNPs in selective NSAID hypersensitivity. In addition, we previously identified genetic factors related to cross intolerance (Agúndez et al., 2012) and, of these, one non-synonymous *DAO* gene variation, designated as rs10156191, was overrepresented among cross-intolerant patients, thus providing the basis for a detailed study on the role of genetic variations in histamine metabolism in patients with selective hypersensitivity to NSAIDs.

FCER1 genotypes have been linked to ASA-intolerant asthma. In a study carried out on 126 Korean patients with ASA-intolerant asthma, Palikhe and co-workers analyzed six

FCER1 SNPs, five of which were also analyzed in our study (Palikhe et al., 2008a,b). They identified a weak association of the *FCER1G* rs11587213 SNP with ASA-intolerant asthma, the patients showing increased frequency for the AA genotype. The same study reported an association of the two *FCER1A* SNPs rs2427827 and rs2251746 with specific IgE levels and an association of the *FCER1G* SNP 11587213 with both total IgE and specific IgE levels.

Our findings do not support an association of these *FCER1A* genotypes with the risk of developing selective hypersensitivity to ASA, or any other NSAID included in this study, and neither do they support an association with total IgE levels. Potential discrepancies between our study and that of Palikhe and co-workers may arise from the differences in clinical presentation, differences in the number of patients, and the different ethnic origin of patients and controls. In fact, in the study by Palikhe et al. (2008a,b) the allele frequencies observed in Korean individuals differ considerably from those reported in this study and in the 1000 genomes website for individuals of Caucasian descent (see **Table 4**).

Additional clinical associations for *FCER1A* SNPs are the putative association of the SNP rs2298804 with the risk of developing systemic lupus erythematosus in a study carried out in China (Yang et al., 2013), and the association of the SNP rs2298805 with the risk of developing chronic spontaneous urticaria in Chinese individuals (Guo et al., 2015). These SNPs are ethnic-specific as they have only been identified in Oriental individuals, but they do not occur in Caucasian individuals according to the 1000 genomes website. Additional clinical associations for *FCER1B* SNPs, all related to the SNP rs569108, include increased risk of developing asthma in Chinese individuals (Ramphul et al., 2014; Hua et al., 2016), and atopic allergy in individuals from Philippines (de Guia et al., 2015). A meta-analysis of 24 studies also supports association of the *FCER1B* rs569108 SNP with asthma, although the risk seems to be restricted to East-Asian individuals (Yang et al., 2014).

Additional clinical associations for *FCER1G* include a weak effect on food sensitization, which is associated with the interactive effect of the *FCER1G* rs2070901 SNP with other SNPs in the *IL4*, *FCER1B,* and *CYP24A1* genes and cord blood 25(OH) D (Liu et al., 2011).

Strengths of this study include a high number of patients with selective NSAID-hypersensitivity ($n = 314$). Moreover, the clinical phenotypes of these patients, including the proportion of each gender, ages, culprit drugs, and patients' clinical presentations correspond to those described previously among Spaniards, thus indicating that the patient group is representative. The number of patients and controls is sufficiently high to obtain a good statistical power, which is required to obtain conclusive evidence. Limitations in this study include a low number of patients for some subgroups according to the culprit drug (**Table 2**), the younger age of control individuals as compared to patients, and the low frequency for some of the SNPs analyzed which were, nevertheless, included in the study because of their functional or clinical impact (**Table 4**).

The results of this study do not support a major association of *FCER1* genotypes in the risk of developing selective NSAID-induced hypersensitivity. Similar genotype distributions and allele frequencies were observed among patients and controls, and the genotypes and frequencies correspond to those previously reported among Spanish patients (Agúndez et al., 2012; Amo et al., 2016). Similarly, no major differences in histamine-metabolizing genes were observed, with the exception of a marginally significant lower frequency of homozygous variant genotypes corresponding to the *DAO* gene variation rs10156191 among patients, which was not significant on comparing allele frequencies (**Table 6**). The rest of the histamine-metabolism SNPs did not show significant differences when patients and controls were compared, and the genotypes and allele frequencies corresponded with those previously reported for Spanish individuals (García-Martín et al., 2006, 2008, 2015b; Agúndez et al., 2012). Although some gender-related risk associations were identified (summarized in **Table 7**), in all cases these were due to differences in allele frequencies, but no statistically significant differences for genotypes, in any of the genetic models analyzed, were identified.

In addition to *FCER1A* genes, we analyzed histamine-metabolism genes because these genes, alone or interacting with *FCER1A* genes, may be involved in, and hence modulate, the events that occur downstream of reaction triggering. Again, to our knowledge no previous studies addressing the role of histamine-metabolism in these genes in selective NSAID hypersensitivity have been carried out. We previously reported that the non-synonymous variant on the diamine oxidase gene, rs10156191, which causes decreased metabolic capacity, was significantly associated with cross-intolerance to NSAIDs (OR, 1.7; 95% CI, 1.3–2.1; $Pc = 0.0003$; (Agúndez et al., 2012)). Conversely, in the present study we did not find any association of this SNP with overall selective hypersensitivity. Regarding *HNMT*, it has been reported that the 939A>G polymorphism, which lowers HNMT enzymatic activity by decreasing HNMT mRNA stability, is associated with aspirin intolerant chronic urticaria (Kim et al., 2009). This aside, no other studies analyzing the possible role of gene-related histamine metabolism in NSAID-hypersensitivity have been published. The lack of association of selective NSAID-hypersensitivity with functional histamine-metabolizing SNPs observed in our study is somewhat unexpected because the culprit NSAIDs implicated in both selective and cross hypersensitivity reactions are the same. However, it should be taken into consideration that the mechanisms involved in cross-intolerance are completely different from those involved in selective hypersensitivity (Agúndez et al., 2012) and this may explain the differences in the linkage of histamine-related genes with the clinical entities.

Phenotype-genotype interaction, however, may be relevant to selective NSAID hypersensitivity: We analyzed by linear regression the putative interaction of all genotypes as well as phenotypic factors. The most relevant phenotypic factor was previous history of atopy, which was strongly related to hypersensitivity to ibuprofen. Age was related to hypersensitivity to ibuprofen. Gender was related to the risk of developing hypersensitivity to ASA, with men showing increased risk, which is unexpected because drug allergy is more frequent

in women (Doña et al., 2011). This drug-specific phenotypic feature deserves further investigation. This study also revealed the association of the *DAO* rs2052129 GG genotype with hypersensitivity to ibuprofen and a weak association of the *DAO* SNP rs10156191 in homozygosity for the detrimental allele (TT) to an increased risk of developing selective hypersensitivity to ASA, but not to other NSAIDs. The fact that we observed a statistically significant association after multiple regression with only 19 patients with selective hypersensitivity to ASA suggests that the association is strong. Although we should be cautious with regard to this association because of the low number of individuals carrying the TT genotype, further studies are warranted. Additional studies focusing on genes known to be related to organ-specific NSAID-induced hypersensitivity, such as hepatotoxicity (Lucena et al., 2008, 2010; Andrade et al., 2009; Agúndez et al., 2011), should also be conducted in subgroups of selective responders to NSAIDs stratified according to the culprit drug.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: EG Performed the experiments: GA and GE. Analyzed the data: JA and EG. Wrote the paper: EG and JA. Patient assessment: MB, JG, CC, JC, MT, CrM, NB, and GC. Acquisition of data: GA, JG, CC, JC, MT, CaM, NB, GC, GE, MB, JA, and EG. Statistical analysis: AR, JA, and EG. All authors participated in the critical review of the manuscript.

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Detoxifying Enzymes at the Cross-Roads of Inflammation, Oxidative Stress, and Drug Hypersensitivity: Role of Glutathione Transferase P1-1 and Aldose Reductase

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Phase I and II enzymes are involved in the metabolism of endogenous reactive compounds as well as xenobiotics, including toxicants and drugs. Genotyping studies have established several drug metabolizing enzymes as markers for risk of drug hypersensitivity. However, other candidates are emerging that are involved in drug metabolism but also in the generation of danger or costimulatory signals. Enzymes such as aldo-keto reductases (AKR) and glutathione transferases (GST) metabolize prostaglandins and reactive aldehydes with proinflammatory activity, as well as drugs and/or their reactive metabolites. In addition, their metabolic activity can have important consequences for the cellular redox status, and impacts the inflammatory response as well as the balance of inflammatory mediators, which can modulate epigenetic factors and cooperate or interfere with drug-adduct formation. These enzymes are, in turn, targets for covalent modification and regulation by oxidative stress, inflammatory mediators, and drugs. Therefore, they constitute a platform for a complex set of interactions involving drug metabolism, protein haptenation, modulation of the inflammatory response, and/or generation of danger signals with implications in drug hypersensitivity reactions. Moreover, increasing evidence supports their involvement in allergic processes. Here, we will focus on GSTP1-1 and aldose reductase (AKR1B1) and provide a perspective for their involvement in drug hypersensitivity.

Keywords: glutathione transferase, aldose reductase, inflammation, oxidative stress, detoxification, allergy, drug adduct, drug hypersensitivity

INTRODUCTION

Drug hypersensitivity reactions pose an important clinical problem. They reduce the therapeutic armamentarium and may entail great severity, being life threatening in some cases. These reactions are mediated by the activation of the immune system by drugs or their metabolites. This can occur through the direct interaction of the drug/metabolite with receptors from immune cells or

by covalent attachment of the drug to endogenous proteins, in a process known as haptenation. It is often considered that drugs are too small structures to activate the immune system on their own, whereas haptenated proteins or peptides can fulfill this role and be processed and presented by antigen presenting cells. In addition, factors leading to the exacerbation of the inflammatory response, the generation of danger signals or oxidative stress, contribute to the development of hypersensitivity reactions through mechanisms not completely understood.

Detoxifying and metabolic enzymes play multiple roles in cell homeostasis and may participate in drug hypersensitivity through various mechanisms. Metabolites produced by drug transformation carried out by these enzymes could activate the immune system. In addition, detoxifying enzymes play important roles in the control of inflammation, cellular redox status, and cytotoxicity.

Inflammation and oxidative stress cooperate in the pathogenesis of allergic diseases. A situation of oxidative stress may concur with sensitization and favor Th2 responses (Utsch et al., 2015). Moreover, oxidative stress induction is common to chemical allergens, including those that induce type IV hypersensitivity (Corsini et al., 2013). Indeed, numerous drugs, including doxorubicin, dapsone, cisplatin, sulfamethoxazole, and many others, elicit oxidative stress through multiple mechanisms (Bhaiya et al., 2006; Deavall et al., 2012; Hargreaves et al., 2016), increasing the generation of danger signals that act as coactivators for the allergic reaction (Sanderson et al., 2006). In turn, oxidative stress can increase the formation of drug-protein adducts by favoring the generation of reactive metabolites of drugs, thus facilitating protein haptenation and subsequent activation of the immune system or other toxic effects. Furthermore, oxidized proteins may be more susceptible to the addition of certain drugs or drug metabolites (Lavergne et al., 2009). Oxidative stress can also alter the ratio between reduced and oxidized glutathione species by depletion of the reduced form (GSH), thus favoring protein glutathionylation and/or reducing the possibility of drug detoxification through GSH conjugation. Conversely, it has been reported that antioxidants such as N-acetylcysteine, ebselen, and pyrrolidine dithiocarbamate can ameliorate immune and allergic responses in several models (Matsue et al., 2003; Monick et al., 2003; Galbiati et al., 2011). Importantly, a reduced antioxidant or cytoprotective capacity has been evidenced in allergy and asthma (Lutter et al., 2015), and sensitization to certain allergens is associated with inadequate antioxidant responses. Consequently, it has been proposed that exploring the master regulator of antioxidant responses Nuclear factor erythroid 2-related factor 2 (Nrf-2), may provide novel biomarkers for determining the sensitization potential of several chemicals (Natsch and Emter, 2008; Ade et al., 2009).

Recently, we have studied two types of detoxifying enzymes, GST and AKR (Sánchez-Gómez et al., 2007, 2010; Díez-Dacal et al., 2016), which interact with several drugs and are important players in the regulation of inflammation and redox status. Indeed, genetic variations in these enzymes have been associated with an increased risk of suffering diseases with an important allergic component such as atopy or asthma. Nevertheless, whereas the role of other drug metabolizing enzymes, such as cytochromes, in drug hypersensitivity has been frequently explored (Gueant et al., 2008; Bhattacharyya et al., 2014), those of GST and AKR remain poorly understood. Here, we provide a perspective on the interactions of GSTP1-1 and AKR1B1 with both drugs and factors contributing to allergic reactions, and suggest avenues to assess their potential as drug hypersensitivity biomarkers.

GSTP1-1

Glutathione-S-transferases are phase II enzymes that detoxify numerous endogenous and exogenous compounds by conjugation with GSH (Hayes et al., 2005). GSH-conjugates can then be exported from cells by the multidrug transporter system (Díez-Dacal and Pérez-Sala, 2012). Numerous genetic variations in GST enzymes have been identified and their functional consequences have been the subject of previous review (Board and Menon, 2013). Regarding GSTP1-1, the polymorphisms described have been mostly studied in the context of cancer and drug metabolism. However, in addition to its metabolic function, GSTP1-1 modulates stress response cascades by mechanisms involving protein-protein interactions with signaling proteins, like c-Jun terminal Kinase (JNK) and other mitogen activated protein kinases, Peroxiredoxin 6 (Prdx6), and Tumor necrosis factor (TNF)-associated factor 2 (TRAF2; Adler et al., 1999; Wu et al., 2006). Moreover, GSTP1-1 facilitates protein glutathionylation, thus regulating protein activity (Tew, 2007). Therefore, a complex landscape appears in which GSTP1-1 integrates cellular responses to redox stress by catalytic, protein-protein interaction and posttranslational mechanisms (**Figure 1**).

Interaction of GSTP1-1 with Oxidative Stress

GSTP1-1 is a key factor for cellular adaptation to oxidative stress at multiple levels. *GSTP1-1* expression is strongly induced by oxidative stress as a defense mechanism through the binding of transcription factors, like Nrf-2 and activator protein (AP)-1, to the antioxidant response elements in its promoter (Kawamoto et al., 2000; Hayes et al., 2005). In turn, oxidative stress can reversibly inactivate GSTP1-1 by intramolecular disulfide formation or oligomerization (Shen et al., 1993; Sánchez-Gómez et al., 2010). Moreover, several electrophilic agents, including endogenous reactive mediators and drugs, induce an irreversible crosslinking of the enzyme (Sánchez-Gómez et al., 2013). The main residues involved in these modifications are the most reactive cysteines in GSTP1-1, namely, Cys47, and/or Cys101. Both, GSTP1-1 oligomerization and crosslinking affect its interactions with signaling proteins and stress cascades, as mentioned above.

GSTP1-1 can promote the reversible incorporation of GSH (S-glutathionylation) into low pKa cysteine residues of proteins. This modification modulates protein function, but also protects cysteine residues from further irreversible oxidations (Tew, 2007; Townsend et al., 2009), allowing the reduced form to

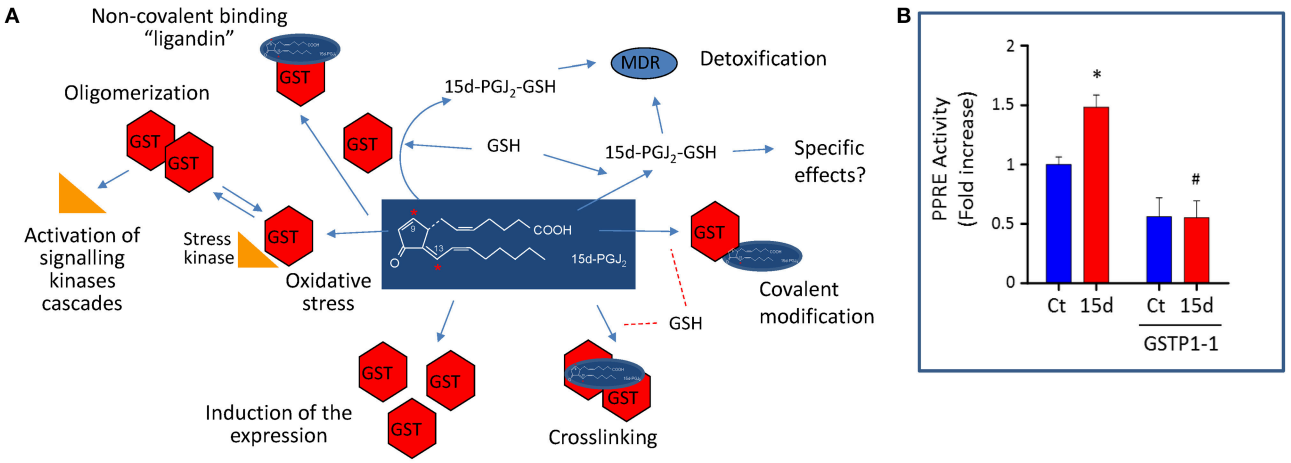

FIGURE 1 | Interactions of GSTP1-1 with the cyPG 15d-PGJ$_2$ as a model of an electrophilic compound that can act both as a substrate and an inhibitor of the enzyme. (A) GSTP1-1 (depicted as "GST" in the scheme) can detoxify electrophilic mediators, like 15d-PGJ$_2$, and drugs, by conjugating them with GSH or through its ligandin role. In turn, GSTP1-1 can be covalently modified and/or cross linked by these compounds. Crosslinking or oligomerization secondary to drug-induced oxidative stress can impact stress signaling cascades. In addition, electrophilic drugs or mediators can induce GSTP1-1 expression in a cell-type dependent manner. **(B)** The ability of GSTP1-1 to detoxify and reduce the effects of 15d-PGJ$_2$ is illustrated: GSTP1-1 overexpression blocks the activity of a PPAR promoter reporter element (PPRE) in cells. Rat mesangial cells were transfected with PPRE as previously described (Zorrilla et al., 2010), and with a GSTP1-1 expression vector where indicated. Then cells were treated in the absence (Ct) or presence of 15d-PGJ$_2$ (15d) and the promoter activity measured by luminescence. The overexpression of GSTP1-1 was sufficient to block PPAR activation induced by the prostaglandin. $^{*}p > 0.05$ vs. Ct, $^{\#}p < 0.05$ vs. 15d-PGJ$_2$. Values represent mean ± SEM from three different experiments.

be regenerated. Proteins S-glutathionylated by GSTP1-1 include Prdx6 (Manevich and Fisher, 2005), AKR1B1, and GSTP1-1 itself (Townsend et al., 2009; Wetzelberger et al., 2010).

Altogether, this evidence illustrates the complex redox regulation of GSTP1-1. Under mild oxidative stress, induction of GSTP1-1 expression and its redox "recycling" function afford cellular protection. However, pharmacological treatments or acute inflammation can inactivate GSTP1-1 either by direct oxidation and/or chemical inhibition. In both cases, allelic variants of GSTP1-1, namely, wild type GSTP1-1 (Ile105, Ala114) and variants: GSTP1-1(Ile105Val, Ala114), GSTP1-1(Ile105Val, Ala114Val), and GSTP1-1(Ile105, Ala114Val), differentially exert protective functions on protein activity and lipid peroxidation, which may influence susceptibility to oxidative stress of subjects carrying the various forms (Manevich et al., 2013).

Interaction of GSTP1-1 with Drugs

GSTP1-1 displays multiple interactions with drugs, either catalyzing their detoxification by GSH conjugation or being inactivated by them. These interactions are crucial for cancer therapy. GSTP1-1 overexpression is an important factor involved in tumor chemoresistance (Díez-Dacal and Pérez-Sala, 2012), and therefore, an important drug target, for which structurally diverse inhibitors, including ethacrynic acid, glutathione analogs, GSTP1-1 activatable drugs, and natural compounds have been considered (Singh, 2015). The mechanism of action of these compounds frequently involves binding to cysteine residues and/or GSTP1-1 oligomerization, as it occurs with electrophilic prostaglandins (PGs) or chlorambucil (Sánchez-Gómez et al., 2013). Interestingly, the pattern of GSTP1-1 crosslinking and/or chemical modifications depends on the presence of both substrates and inhibitors, for which this enzyme can be considered a converging platform for the effects of drugs and danger signals arising from oxidative stress or inflammation (Sánchez-Gómez et al., 2013).

GSTP1-1 also keeps important direct or indirect interactions with the mechanism of action of drugs such as acetaminophen (McGarry et al., 2015), acetylsalycilic acid (Baranczyk-Kuzma and Sawicki, 1997), and other non-steroidal anti-inflammatory drugs (Orhan and Sahin, 2001). In fact, GSTP1-1 deficiency correlates with higher acetaminophen toxicity in mice (McGarry et al., 2015). Also, a "ligandin" role of GSTP1-1 should be taken into account, since this abundant cytosolic enzyme can sequester drugs, thus reducing their effective concentrations (Oakley et al., 1999; Lu and Atkins, 2004).

Interaction of GSTP1-1 with Inflammatory Mediators

GSTP1-1 also displays multiple interactions with inflammation: it is induced by proinflammatory stimuli, but this could exert a negative feedback on the inflammatory response. GSTP1-1 ameliorates the inflammatory response in several experimental models of tissue damage or inflammation (Xue et al., 2005; Luo et al., 2009). Interestingly, several GST, including GSTP1-1, attenuate the action of the inflammatory mediator 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$; Paumi et al., 2004). Evidence from our laboratory indicates that overexpression of GSTP1-1 in rat mesangial cells reduces the capacity of 15d-PGJ$_2$ to activate Peroxisome proliferator activated receptor (PPAR) transcription factor(s) (**Figure 1**). Moreover, a reduction in the

basal PPAR activity is also observed, suggesting the inactivation of endogenous PPAR agonists or the participation of additional mechanisms in GSTP1-1 regulation of inflammation.

In turn, electrophilic mediators like 15d-PGJ_2 can inhibit GST activity in several cell types through various mechanisms (Sánchez-Gómez et al., 2007). Interestingly, cyclopentenone prostaglandins (cyPG) with dienone structure induce an extensive intermolecular crosslinking of GSTP1-1 monomers, involving mainly Cys47 and Cys101 (Sánchez-Gómez et al., 2013) that is blocked by GSH or non-metabolizable GSH analogs, indicating that cyPG-GSTP1-1 interaction is impaired in the GSH-bound enzyme.

Therefore, the interaction of GSTP1-1 with inflammatory mediators like cyPG is a two-way process strongly dependent on GSH availability (Gayarre et al., 2005; Díez-Dacal and Pérez-Sala, 2010), since the enzyme can conjugate electrophilic mediators with GSH, whereas cyPG can induce the expression and/or inhibit GST activity in a cell type-dependent manner (Sánchez-Gómez et al., 2007). Some of these interactions have also been evidenced for other GST isoforms (Gilot et al., 2002; Kudoh et al., 2014). These observations illustrate the intricate implications of GST in inflammation, with the net outcome depending on the delicate balance of all these factors.

GSTP1-1 in Allergic Reactions

Although GST have been mostly studied in the fields of oxidative stress and chemoresistance, an interesting role in allergic reactions is emerging. Endogenous GSTP1-1 is an important target for haptenation, which has been related to the induction of certain drug hypersensitivity reactions (Meng et al., 2014). In addition, genetic variants of several GST isoforms have been found to associate with allergic processes including asthma (Tamer et al., 2004), drug eruptions (Ates et al., 2004), sensitization to thimerosal (Westphal et al., 2000), or allergic rhinitis (Iorio et al., 2014). In the case of GSTP1-1, both down- and up-regulations of GSTP1-1 levels have been reported in association with asthma (Schroer et al., 2011): whereas low levels could contribute to asthma, oxidative stress associated with the allergic response could induce GSTP1-1 expression. These changes in expression may in turn be modulated by the occurrence of polymorphisms, like Ile105Val (rs 1695; Dragovic et al., 2014), since this variant has been reported to display a reduced ability to conjugate several electrophilic drugs and reactive metabolites to GSH, and may associate with certain allergic diseases, including atopy and asthma (Hoskins et al., 2013). Polymorphic forms of GSTP1-1 correlate with the aggravation of asthma symptoms induced by air pollution (Su et al., 2013), and increased risk of asthma associated with acetaminophen (Kang et al., 2013) and exercise (Islam et al., 2009). In addition, the Ile105 wild type enzyme associates with enhancement of certain nasal allergic responses (Gilliland et al., 2004), whereas, according to another study, the Ala114 wild type enzyme associates with increased risk of atopy (Schultz et al., 2010). Nevertheless, lack of association of GSTP1-1 polymorphisms with allergic diseases or drug hypersensitivity has been reported in other studies, potentially due to differences in the genetic backgrounds of the patient cohorts studied.

Altogether, these findings support the role of GSTP1-1 as a risk factor in hypersensitivity responses by multiple mechanisms, given its multifunctional involvement in drug metabolism and inflammation. Moreover, GSTP1-1 emerges as a key factor to be considered in future genomic studies related with allergy development and drug hypersensitivity reactions.

AKR1B1

AKR1B1 (or aldose reductase) is a member of the AKR superfamily, which comprises multiple enzymes involved in oxidoreduction of endogenous and exogenous compounds, including aliphatic and aromatic aldehydes, monosaccharides, steroids, aromatic hydrocarbons (PAHs), or isoflavonoids, using NADH or NADPH as cofactors. Structurally, this phase I metabolizing enzyme (Penning and Drury, 2007) is folded into a $(\alpha/\beta)_8$-barrel motif that is highly conserved among the members of this family and harbors the active site at its C-terminal end (Jez et al., 1997).

AKR1B1 primary role is to afford constitutive and inducible protection against toxic aldehydes generated under oxidative stress (Jin and Penning, 2007; Lyon et al., 2013). AKR1B1 reduces highly reactive lipid peroxidation products like 4-hydroxy-2-nonenal (HNE), acrolein, and methylglyoxal, as well as GSH-conjugates of these aldehydes such as glutathionyl-4-hydroxy-2-nonenal (GS-HNE) and GS-acrolein (Kolb et al., 1994; Srivastava et al., 1998; Vander Jagt et al., 2001). For instance, AKR1B1 activation played a cardioprotective role in rat myocardial ischemia by decreasing the accumulation of lipid peroxidation products in the ischemic heart (Kaiserova et al., 2008). Similarly, induction of AKR1B1 expression in response to oxidative stress plays a role in the antioxidant response (Wang et al., 2012). AKR1B1 also participates in steroid hormones catabolism and plays an important role in the regulation of steroid function in several tissues (Barski et al., 2008).

Nevertheless, AKR1B1 also has a negative side since it can promote tumor chemoresistance and contribute to the perpetuation of inflammation and to the development of secondary diabetic complications (**Figure 2**).

Interactions of AKR1B1 with Oxidative Stress

AKR1B1 activity is regulated by oxidative posttranslational modifications. The highly nucleophilic Cys298, located near the active site, can be modified by different reactive species like nitric oxide (NO), HNE, or oxidized glutathione. These modifications may reduce or increase AKR1B1 catalytic activity, depending on the modifying moiety, and reduce its susceptibility to pharmacological inhibitors. Interestingly, NADPH protects Cys298 from modification by these agents (Chandra et al., 1997; Del Corso et al., 1998; Petrash, 2004).

AKR1B1 is a target gene of Nrf-2, the master transcription factor regulating the antioxidant response. Therefore, it is induced by numerous oxidative stimuli and participates in the antioxidant response (Kang et al., 2007; Wang et al., 2012). In consequence, AKR1B1 expression is increased in tissues

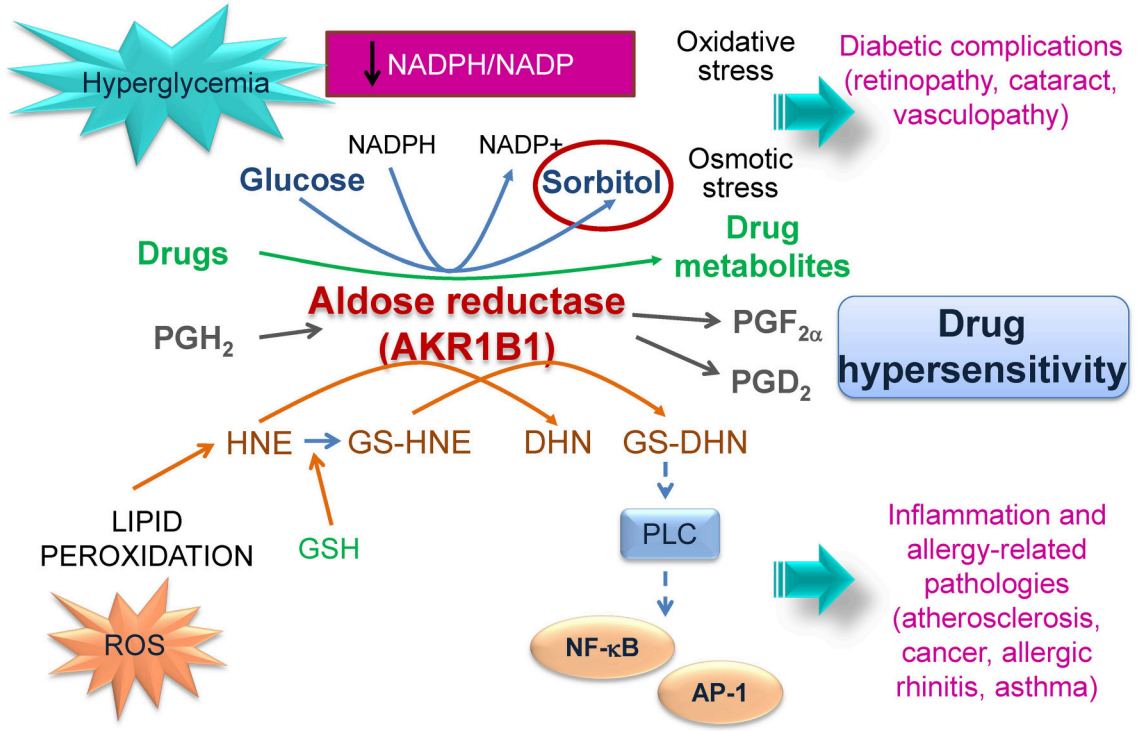

FIGURE 2 | Involvement of AKR1B1 in pathophysiology. AKR1B1 catalyzes the first step in the polyol pathway transforming glucose into sorbitol using NADPH as cofactor. Under hyperglycemic conditions increased sorbitol and NADPH consumption lead to osmotic and oxidative stress, respectively, that can contribute to diabetic complications. AKR1B1 metabolizes drugs leading to inactivation and chemoresistance and/or to the generation of toxic metabolites. In addition, AKR1B1 can metabolize PGH_2 yielding $PGF_{2\alpha}$, which may regulate PGE_2 production. Transformation of reactive aldehydes or their GSH-conjugates by AKR1B1 can generate species that perpetuate inflammation and may be involved in allergic responses. The interactions of AKR1B1 with drug metabolism, oxidative stress, inflammation an allergic reactions support its consideration in studies of drug hypersensitivity.

with elevated oxidative stress, e.g., in alcoholic liver disease or vascular inflammation (Srivastava et al., 2005), where in some cases affords a protective role (Kang et al., 2014). Nevertheless, excessive AKR1B1 activity can lead to NADPH depletion and oxidative stress.

This occurs in diabetes, where AKR1B1 metabolizes excess glucose through the polyol pathway. An increased flux through this pathway can lead to osmotic stress due to the increased formation of sorbitol, as well as to a redox imbalance by the elevated consumption of NADPH (Petrash, 2004; **Figure 2**). NADPH is a substrate/cofactor for several enzymes involved in the cellular antioxidant defense, including glutathione reductase (GSH regeneration), peroxiredoxins and thioredoxin, as well as for several detoxifying systems (Pollak et al., 2007a). Therefore, depletion of NADPH changes the NADPH/$NADP^+$ ratio contributing to oxidative stress and reducing the cellular ability to recover after an oxidative insult (Pollak et al., 2007b; Ying, 2008).

Interaction of AKR1B1 with Drugs

AKR1B1 is an important drug target due to its implication in the development of diabetic complications. Therefore, the search for inhibitors from both synthetic and natural sources has yielded a wide array of compounds that bind and/or inhibit the enzyme, with structural information on their binding arising from molecular modeling or crystallographic studies. AKR enzymes are involved in chemoresistance because they metabolize carbonyl-containing drugs, including naloxone and ketotifen (Endo et al., 2014). The anthracycline antibiotics doxorubicin and daunorubicin pose an important case, since they are among the most effective chemotherapic drugs. However, the reduction of their carbonyl group to their corresponding alcohol, yielding doxorubicinol and daunorubicinol, respectively, reduces their efficacy (Veitch et al., 2009). Overexpression of AKR1B1 inactivates these drugs and leads to resistance of various tumor cells (Plebuch et al., 2007; Heibein et al., 2012). Conversely, AKR1B1 inhibition increases the cytotoxic effects of the anticancer agents doxorubicin and cisplatin in HeLa cervical carcinoma cells (Lee et al., 2002), and the AKR inhibitors PGA_1 and AD-5467 improve the effectiveness of doxorubicin in lung cancer cells (Díez-Dacal et al., 2011; Díez-Dacal and Pérez-Sala, 2012). Natural variants of certain AKR enzymes have been identified that present a reduced capacity to metabolize daunorubicin and doxorubicin *in vitro* (Bains et al., 2008, 2010). There is little information on the involvement of AKR1B1 metabolites in hypersensitivity reactions. Nevertheless, daunorubicinol has toxic effects *per se* because it induces cardiomyopathy (Minotti et al., 2004).

Interaction of AKR1B1 with Inflammatory Mediators

AKR1B1 plays an important role in different inflammatory diseases such as atherosclerosis, sepsis, asthma, uveitis, and colon cancer. AKR1B1 can be induced by proinflammatory stimuli (Bresson et al., 2012). Transcription factors Nuclear factor (NF)-κB and AP-1 activate the AKR1B1 promoter through binding to the osmotic response element (ORE; Iwata et al., 1997; Lee et al., 2005) and the phorbol ester response or AP-1 sites, respectively (Penning and Drury, 2007).

Although AKR1B1 can play a protective role by detoxifying acrolein or HNE, it can also play a positive/amplifying role in inflammation through various mechanisms (**Figure 2**). In particular, metabolism of HNE or its glutathione conjugate GS-HNE can result in products, such as 1, 4-dihydroxynonene (DHN) and glutathionyl-1,4-dihydroxynonane (GS-DHN), which are still toxic and promote activation of phospholipase C (PLC)-NF-κB cascades perpetuating inflammation (Ramana et al., 2006; Srivastava et al., 2011). Thus, inhibition of AKR1B1 reduced NF-κB-dependent inflammatory markers, and the synthesis of TNF-α stimulated by hyperglycemic conditions, and of inflammatory mediators like NO and PGE_2 (Ramana and Srivastava, 2010).

Interestingly, AKR1B1 displays PGF2α synthetizing activity through which it can regulate PGE_2 production (Bresson et al., 2012), thus contributing to the modulation of inflammation. In turn, AKR1B1 can bind several PG, including PGE_1 and PGE_2 and their cyclopentenone products, PGA_1 and PGA_2, which results in inhibition of the enzyme (Díez-Dacal et al., 2016). However, whereas binding and inhibition by PGE appear to be fully reversible, cyPG form a Michael adduct that seems irreversible under certain conditions. Nevertheless, concentrations of GSH in the cellular range (millimolar) elicit a retro-Michael reaction, a fact that contributes to explain the more intense modification and inhibition of some AKRs detected in GSH-depleted cells (Díez-Dacal et al., 2011).

AKR1B1 in Allergic Reactions

Early reports linking AKR1B1 to hypersensitivity provided fragmented pieces of evidence. The AKR1B1 inhibitor sorbinil, not currently used in clinical practice, elicited severe adverse effects, including hypersensitivity attributed to protein adducts produced by sorbinil metabolites (Maggs and Park, 1988). Interestingly, lodoxamide tromethamine, and several anti-allergy drugs, inhibit AKR1B1 (White, 1981), providing additional possibilities of interaction with the hypersensitivity response.

Recent studies using pharmacological or genetic depletion establish a positive role for AKR1B1 in allergy. In mice, AKR1B1 inhibition reduced airway inflammation, hyperresponsiveness and IgE and Th2-cytokine levels in ovalbumin and ragweed pollen extract-induced asthma (Yadav et al., 2009, 2011a). Furthermore, studies in $AKR^{-/-}$ mice also support a role of AKR1B1 in the pathogenesis of asthma and allergic rhinitis (Yadav et al., 2011b, 2013a). Moreover, the efficacy of AKR1B1 inhibitors in mouse models supports their use to treat these allergic conditions (Yadav et al., 2011b, 2013a). In mice sensitized with ovalbumin, AKR1B1 inhibition with fidarestat prevented the airway remodeling observed in chronic asthma by blocking the tumor growth factor β (TGFβ), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Protein kinase B (PKB/AKT)/Glycogen synthase kinase-3 beta (GSK3B) axis (Yadav et al., 2013b).

The mechanisms linking AKR1B1 with allergy are not fully understood. Nevertheless, it could be hypothesized that it provides coactivators of the allergic response through its contribution to oxidative stress or to the generation of proinflammatory mediators, like aldehyde conjugates.

In contrast to the numerous studies on GSTP1-1 polymorphisms in allergic patients, most genetic studies on AKR1B1 have been directed to explore its association with the development of diabetic implications (Demaine, 2003), and very little information exists on the impact of AKR1B1 variants on drug metabolism or hypersensitivity reactions. Nevertheless, given the fact that an increased glucose flux through the polyol pathway leads to redox imbalance, it would be interesting to assess the involvement of AKR1B1 variants in oxidative stress. In addition, the recent evidences on the involvement of AKR1B1 in allergy grant its study in association with these processes.

In summary, AKR and GST enzymes are emerging as important regulators of the balance of inflammatory mediators. This, together with their association with allergic processes and their ability to metabolize and be covalently modified by drugs makes them attractive candidates to explore their involvement not only in allergy in general but in drug hypersensitivity.

AUTHOR CONTRIBUTIONS

FS contributed to manuscript writing, figure preparation and experimental work. BD contributed to manuscript writing and figure preparation. EG contributed to manuscript writing. JA contributed to manuscript writing. MP contributed to manuscript writing. DP coordinated and wrote the manuscript and prepared figures.

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REVIEW
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In vitro Models to Evaluate Drug-Induced Hypersensitivity: Potential Test Based on Activation of Dendritic Cells

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Hypersensitivity drug reactions (HDRs) are the adverse effect of pharmaceuticals that clinically resemble allergy. HDRs account for approximately 1/6 of drug-induced adverse effects, and include immune-mediated ("allergic") and non-immune-mediated ("pseudo allergic") reactions. In recent years, the severe and unpredicted drug adverse events clearly indicate that the immune system can be a critical target of drugs. Enhanced prediction in preclinical safety evaluation is, therefore, crucial. Nowadays, there are no validated *in vitro* or *in vivo* methods to screen the sensitizing potential of drugs in the pre-clinical phase. The problem of non-predictability of immunologically-based hypersensitivity reactions is related to the lack of appropriate experimental models rather than to the lack of -understanding of the adverse phenomenon. We recently established experimental conditions and markers to correctly identify drug associated with *in vivo* hypersensitivity reactions using THP-1 cells and IL-8 production, CD86 and CD54 expression. The proposed *in vitro* method benefits from a rationalistic approach with the idea that allergenic drugs share with chemical allergens common mechanisms of cell activation. This assay can be easily incorporated into drug development for hazard identification of drugs, which may have the potential to cause *in vivo* hypersensitivity reactions. The purpose of this review is to assess the state of the art of *in vitro* models to assess the allergenic potential of drugs based on the activation of dendritic cells.

Keywords: CD86, ROS, alternative methods, drug hypersensitivity, *in vitro* methods, dendritic cell activation

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INTRODUCTION

Adverse drug reactions (ADRs) are defined by the World Health Organization as "any noxious, unintended, and undesired effect of a drug that occurs at doses used for prevention, diagnosis, or treatment". ADRs can be categorized into type A—predictable (about 80% of all ADRs), and type B—unpredictable, reactions. Type A (predictable) reactions are usually dose-dependent, related to the known pharmacologic actions of the drug, and occur in otherwise health subject while type B (unpredictable) reactions are generally dose independent, are unrelated to the pharmacologic actions of the drugs, and occur only in susceptible subjects. Unpredictable reactions are subdivided into drug intolerance, drug idiosyncrasy, drug allergy and pseudo-allergic reactions (Khan and Solensky, 2010). It is difficult to distinguish between pseudo allergic reactions and true immunologically mediated allergic reactions, but

the first one lack immunological specificity (Warrington and Silviu-Dan, 2011). The Gell and Coomb's classified hypersensitivity reactions into 4 types and this classification system includes:

- Type I reactions: immediate-type reactions mediate by immunoglobulin E (IgE) antibodies. Drug IgE complex bind to mast cells with release of histamine and inflammatory mediators, resulting in anaphylaxis, urticarial, angioedema, bronchospasm. Examples of drugs include penicillin and cephalosporins.
- Type II reactions: cytotoxic reactions mediated by drug-specific immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies. Specific IgG or IgM antibodies are directed at drug-hapten coated cells, resulting in anemia, cytopenia, thrombocytopenia. Examples of drugs includes hydralazine, methyldopa and procainamide.
- Type III: immune-complex reactions. Tissue deposition of drug-antibody complexes result in complement activation and inflammation (i.e., serum sickness, vasculitis, fever, rash, arthralgia, lupus). Examples of drugs include penicillin, sulphonamides, hydralazine and procainamide.
- Type IV reactions: delayed-type hypersensitivity reactions mediated by cellular immune mechanisms. MHC presentation of drugs to T cells results in the release of cytokine and inflammatory mediators, which recruit inflammatory cells (i.e., contact sensitivity, skin rashes, organ-tissue damage). Type IV reactions can be divided in subcategories, with the activation and recruitment of monocytes, eosinophils, CD4+ or CD8+ T cells, and neutrophils (Pichler, 2003a; Riedl and Casillas, 2003; Warrington and Silviu-Dan, 2011). Examples of drugs include neomycin, penicillin and benzocaine.

In vivo tests like patch, prick and intra-cutaneous tests often do not yield positive reactions for the diagnosis of drug allergy and lacks optimal sensitivity that still remains a major problem in daily clinical practice (Sachs et al., 2002). For these reasons, *in vitro* stimulation could be required as a complementary diagnostic test, as emerged from some studies (Torres et al., 2003; Romano et al., 2004; Sachs et al., 2004).

Nowadays there are no validated *in vivo* or *in vitro* methods for assessing the sensitizing potential of a drug during the pre-clinical phase, although the important adverse reactions directly linked to immune-mediated hypersensitivity and autoimmunity reactions. Available *in vitro* tests mainly refer to the effector phase of immediate-type drug allergic reactions, such as the CAST-ELISA® (Kubota et al., 1997), which is based on the presence of specific IgE antibodies and the BASO-Test® (Pâris-Köhler et al., 2000), based on the activation of basophils like.

Currently, the popliteal lymph node assay (PLNA), or its modifications, can be used in research studies for the identification of drugs, which may be potential allergens, or may cause autoimmunogenic reactions (Warbrick et al., 2001). However, no reliable models or general strategy and assays (including the PLNA) are at present available (or validated) and requested by regulatory agencies. PLNA appears to be very useful for the assessment of the potential of drug to initiate an immune response. The simplest, the primary PLNA, measures popliteal lymph node hyperplasia after subcutaneous injection of a chemical into the footpad of a mouse or rat. The PLN-index is obtained with the ratio of weight or cell number of the draining lymph node of the chemical-treated animals over the vehicle-treated animals (Pieters, 2001). With the primary PLNA, the involvement of specific T cells cannot be assessed; therefore, in a previously sensitized animal, a secondary PLNA must be performed by measuring the PLN index of a chemical. In this assay, purified and irradiated T cells from sensitized syngeneic donors are injected subcutaneously into the footpad of naïve acceptor mice 1 day before injection of the chemical or its active metabolite. Finally, the modified PLNA, defined reporter antigens TNP-OVA (T cell-dependent antigen) and TNP-Ficoll (T cell-independent antigen) are used to distinguish between sensitizing and non-sensitizing (IgG1-response or not to TNP-Ficoll, respectively) drugs (Albers et al., 1997). The primary PLNA is particularly suitable as preclinical screening assay, but it cannot distinguish between strong irritants and sensitizing compounds. Also the more complicated modified PLNA may be used as screening assay, and it has additional advantages over the primary PLNA including (a) the parameters measured (antibody production) are immunologically more relevant than lymph node weight or cell number; (b) the immune response can be measured without knowing the nature of the neo-antigens and it can discriminate between sensitizing, non-sensitizing and complete innocent chemicals. In any case, some of the compounds known to cause immune adverse effects in humans, however, failed to induce a positive PLNA response, leading to refinements of the technique to include pretreatment with enzyme inducers, depletion of CD4+ T cells or additional endpoints such as histological examination, lymphocyte subset analysis and cytokine fingerprinting (Ravel and Descotes, 2005).

It is well known that immunological adverse drug reactions are rare (Gruchalla, 2001; Pichler, 2003b) but anyway they are generally able to cause a lot of discomfort to patients and may indeed be really dangerous. The withdrawal from the market of drug is also an important economic issue due to the extremely high costs associated with the development of a drug (Pieters, 2007). The development of alternative *in vitro* assays to detect the sensitization potential during the development phase of a drug would increase safety and possibly reduces the risk of market withdrawal (Corti et al., 2015).

Traditional drugs have low molecular weights (<1000 Da) and as a such they are too small to be "seen" by T cells. Therefore, low molecular weight compounds have first to bind to a protein before they will become visible to T cells (Weltzien et al., 1996; Pichler, 2002). In addition, drugs may alter protein structure, the process of antigen presentation, or may, by causing cellular or organ damage, release auto-antigens (e.g., DNA or histones) for which no tolerance exists (Pieters, 2007), favoring the development of hypersensitivity reactions.

This review will focus on the state of the art of available *in vitro* models to assess the potential of drugs to induce hypersensitivity

for diagnostic purposes and on the potential *in vitro* test for the pre-clinical assessment.

STATE OF THE ART OF *IN VITRO* MODELS TO ASSESS DRUG-INDUCING HYPERSENSITIVITY

The cells involved and mediators released during the different phases of hypersensitivity reactions can be assessed using *in vitro* diagnostic tests. The methods used for the diagnosis of HDR depend on the mechanism involved and the kinetic of the reaction. As shown in **Figure 1**, *in vitro* diagnostic tests can be divided in test able to identify the drugs but only at the resolution of the hypersensitivity reaction, and in *in vitro* assays, which allow to determining the HDR risks before drug administration.

Is important to mention that *in vitro* tests for the identification of non-immediate reactions (NIR) are not commercially available and therefore standardization is not possible (Mayorga et al., 2016).

IN VITRO DIAGNOSTIC TESTS

HLA-Allele Determination

The human leukocyte antigen (HLA) system is a gene complex encoding the major histocompatibility complex (MHC) proteins in humans. HLA genes are highly polymorphic, which means that they have many different alleles, allowing them to fine-tune the adaptive immune system. HLA genotyping is based on reverse sequence-specific oligonucleotide-polymerase chain reaction using DNA from peripheral blood.

Pharmacogenetic testing is not widely used in routine clinical practice to optimize drug choice or clinical management (Philipps, 2006). This gap between scientific knowledge and clinical application may be explained by the fact that the successful incorporation of a pharmacogenetic test into routine practice requires a combination of high-level evidence that can be generalized to diverse clinical setting, wide-spread availability of cost-effective and reliable laboratory tests, and effective strategies incorporate testing into routine clinical practice (Mallal et al., 2008).

Several clinical studies, as reported in the review by Mayorga et al. (2016), correlate hypersensitivity reaction caused by pharmaceuticals with the presence of different HLA allele. In particular HLA-B*57:01 has been found to be associated with abacavir hypersensitivity; for carbamazepine, the most important association has been established with HLA-B*15:02 and HLA-A31:01, while HLA-B*58:01 allele has been associated with allopurinol hypersensitivity.

Prospective HLA-allele screening may be widely useful, but there are some controversial points like the cost-effectiveness of the test, that depend on several estimates that vary among populations, the health care setting, and also the availability of appropriate laboratory assays (Martin et al., 2006; Hammond et al., 2007).

Lymphocyte Transformation Test (LTT)

The LTT measures the *in vitro* proliferative response of T cells that emerge from the clonal expansion of naïve T cell, after drug

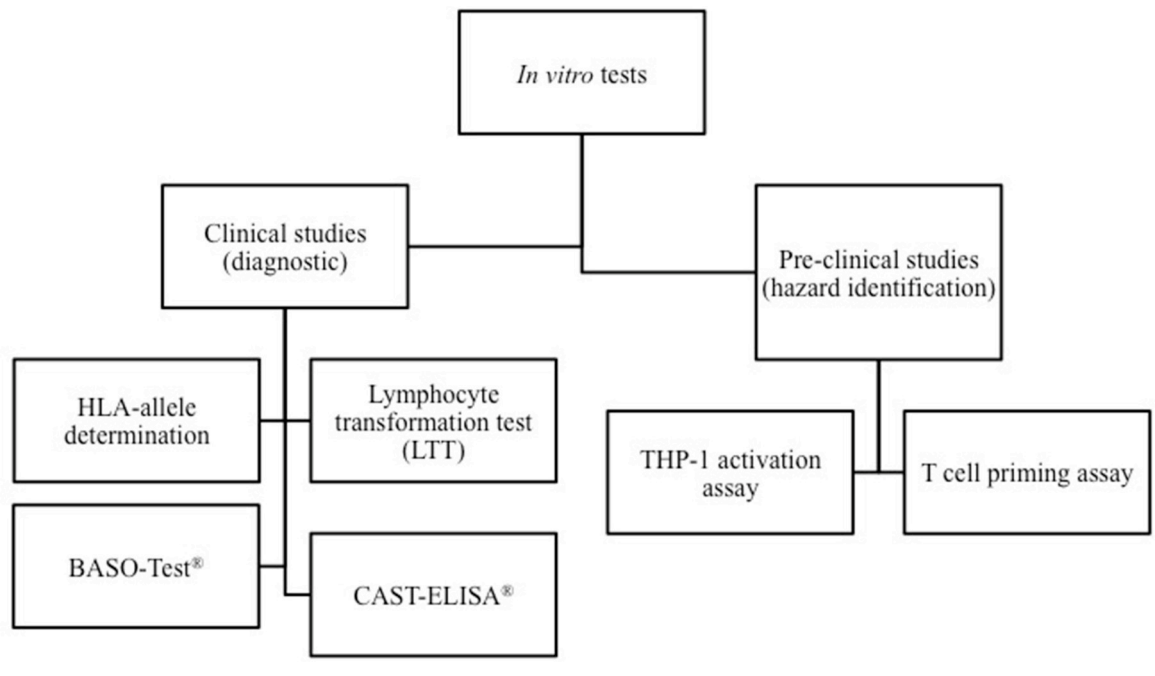

FIGURE 1 | Classification of *in vitro* models able to assess drug-inducing hypersensitivity reactions. *In vitro* diagnostic tests can be divided in test able to identify the culprit drugs at the resolution of the reaction, and methods that allow to determining the individual risk of HDR before drug administration. On the other hand, some *in vitro* methods may be used in the pre-clinical phase of drug development for hazard identification of potential to induce hypersensitivity reactions.

exposure (Nyfeler and Pichler, 1997; Lanzavecchia and Sallusto, 2000). The proliferative response of lymphocytes is measured by the incorporation of ^{3}H-thymidine during DNA synthesis or by carboxyfluorescein diacetate succimidyl ester (CFSE). The most widely studied drugs are betalactams (Mayorga et al., 2016). The LTT limits are represented by the use of radioactivity, which limits the application of the method to specific research laboratories, it is a quite long procedure, the sensitivity is quite low (60–70%), and it depends upon the conditions employed (Nyfeler and Pichler, 1997; Romano et al., 1997; Beeler and Pichler, 2007).

Enzyme-Linked Immunosorbent Spot (ELISpot) Assay

ELISpot is a technique used to determine the number of cells able to produce cytokines and cytotoxic markers after their activation by the drug or its metabolites (Sullivan et al., 2015). Clinical studies report the use of IFN-γ ELISpot to diagnose non-immediate reactions to betalactames; granzyme B and granulysin ELISpot for evaluating severe cutaneous reactions induced by amoxicillin, ciprofloxacin, carbamazepine, sulphonamides, allopurinol, mefenamic acid, oxipurinol, and lamotrigine (Zawodniak et al., 2010; Porebski et al., 2013). One advantage of this *in vitro* test is that drug-reactive T cells remain detectable for long time after the reaction and could be appropriate for high throughput screening but to improve the accuracy of the test, two or more cytokines determination could be necessary.

Cell Markers and Cytokine Release

After drug stimulation, T-cells express or up-regulate a number of surface molecules and produce different inflammatory mediators. Cytokine expression and secretion can be evaluated by several methods such as reverse transcription polymerase chain reaction (RT-PCR), flow cytometric analysis, and enzyme linked immunosorbent assay (ELISA). RT-PCR is used to measure cytokine at the transcriptional levels, while flow cytometric analysis are used to study intracellular cytokines and cell surface markers. Finally, ELISA is used to measure the amount of secreted cytokines in cell culture supernatants (Khalil et al., 2008).

Sachs et al. demonstrated that accumulation of eosinophils following IL-5 secretion, and to a lesser extent also with IL-10 and IFN-γ, from drug-specific stimulated T cells is a characteristic histological feature of drug-induced skin eruption. *In vitro* determination of drug-specific IL-5 secretion by peripheral blood mononuclear cells may be relevant for the detection of in drug-induced maculopapular exanthems (Sachs et al., 2002). CD69 is up-regulated after 48–72 h, and its determination by flow cytometry correlates with LTT for betalactames, sulphamethoxazole and carbamazepine HDR (Beeler et al., 2008). CD69 may be used for the evaluation of non-immediate reactions.

These *in vitro* tests represent important tools for diagnosis. They are, however, used mainly as research methods rather than as routine procedures. It must be also taken into consideration the timing of sample collection, which is critical as mediators can be secreted in transitory peaks with variants in the maintenance of detectable levels (Mayorga et al., 2006), and chemokines and cytokines can be degraded by protease (Niwa et al., 2000).

Other *In vitro* Tests

Other *in vitro* tests have been proposed for the study of drug allergy reactions. Among these the cellular allergen stimulation test (CAST-ELISA®) for the measurement of leukotrienes after peripheral blood leukocyte stimulation, basophil histamine release tests and a basophil activation test (BASO-Test®) can be mentioned. CAST-ELISA® is commercially available but it has not been sufficiently evaluated to recommend as a standard investigation outside the context of prospective studies. Basophil activation markers using fluorescence activated cell sorter analysis are currently being evaluated for certain type of drug allergic reactions but there seems to be no evidence currently of any advantage of these tests over skin testing (Mirakian et al., 2009).

POTENTIAL *IN VITRO* PRE-CLINICAL TESTS TO ASSESS HYPERSENSITIVITY

None of the *in vitro* methods mentioned above are, however, useful in preclinical safety assessment. It will important to have *in vitro* methods to screen drugs for their potential to induce hypersensitivity reactions. In the last decade an incredible progress has been made in the development of non-animal tests to assess contact hypersensitivity, and some tests have been formally validated. Methods based on the use of dendritic cells and the T cell priming are discussed below.

The T Cell Priming Assay

Chemicals can elicit T-cell-mediated diseases, including adverse drug reactions. The T cell priming assay (TCPA) was developed primarily for the identification of contact allergens within the integrated EU project SENS-IT-IV. This assay allows the detection of chemical-specific T cells in naive human peripheral T-cell population by measurement of proliferation and, at the single cell level, of IFN-γ production in CD4+ and CD8+ T cells. This assay may be a valuable, highly specific element in an integrated testing strategy for the predictive *in vitro* identification of contact allergens and possibly drugs that cause T cell-mediated adverse drug reactions (Dietz et al., 2010; Martin et al., 2010; Richter et al., 2013).

Myeloid U937 Skin Sensitization Test (MUSST) and Modified MUSST (mMUSST)

The MUSST is an *in vitro* method proposed to assess skin sensitization. Dendritic cell activation following exposure to sensitizers was modeled in the U937 human myeloid cell line by measuring the induction of the expression of CD86 by flow cytometry after 48 h of chemical treatment. A test substance is predicted to have a dendritic cell activating potential indicative of being a sensitizer when CD86 induction exceeds the threshold of 1.5-fold with respect to vehicle treated cells at any tested concentration showing a cell viability ≥70

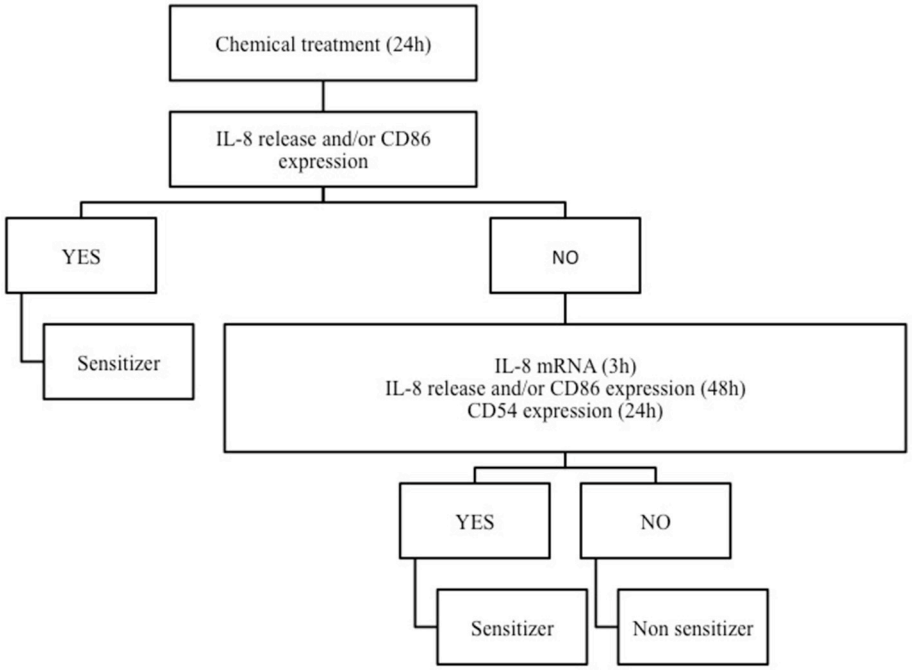

FIGURE 2 | The THP-1 activation assay tier approach. Following 24 h of THP-1 chemical/drug treatment, the effect on IL-8 release and CD86 expression are investigated. If positive (statistically significant release of IL-8 at any concentration and/or a SI $\geq$ 1.5 for CD86), the chemical/drug will be considered as sensitizer. If negative, in order to exclude any activation, IL-8 release and CD86 expression at 48 h (statistically significant release of IL-8 at any concentration and/or a SI $\geq$ 1.5 for CD86) or CD54 expression at 24 h (SI $\geq$ 2.0) or alternatively IL-8 mRNA expression ($2^{-\Delta\Delta CT} > 3.0$) at 3 h should be assessed. Only if negative results were obtained in all parameters, the chemical/drug will be considered as non-sensitizer.

TABLE 1 | Time of IL-8 release and CD86 expression of the selected drugs in THP-1 assay.

Chemical	CV_{75} (μg/ml)	Statistical significant IL-8 release	CD86 expression
Streptozotocin	>2000	24 h	24 h
Sulfamethoxazole	>1000	48 h	48 h
Procainamide	>2000	24 h	24 h
Ofloxacin	>1000	24 h	24 h
Neomycin	>2000	24 h	–
Clonidine	750	24 h	24h
Methyl salicylate	>1000	24 h	24h
Probenecid	600	24 h	48h
Metformin	>2000	–	–

10^6/ml cells were treated for 24–48 h with increasing concentrations of the selected drugs. Cell viability was assessed by PI staining. CV75 (the concentration resulting in 75% of cells viability compared to vehicle treated cells) was calculated by linear regression analysis of data. IL-8 release was measured by ELISA. CD86 expression was evaluated by flow cytometric analysis. Original data are present in Corti et al. (2015).
Legend: – no induction observed.

% in at least two independent experiments (Urbisch et al., 2015).

In the modified version of the MUSST (mMUSST), a test substance is predicted to have a dendritic cell activating potential when CD86 induction exceeds a threshold of 1.2-fold (Bauch et al., 2012). Among more than 145 substances available from these two *in vitro* assays, also some drug sensitizers, namely benzocaine, hydroquinone, p-benzoquinone and diphenylclopropenone were tested and resulted correctly classified with the MUSST (Bauch et al., 2012; Natsch et al., 2013; Urbisch et al., 2015).

The Human Cell Line Activation Test (h-CLAT)

The h-CLAT quantifies changes in CD86 and CD54 expression in the human THP-1 cell line following 24 h exposure to the test chemical. The changes in surface marker expression are measured by flow cytometry. Cytotoxicity measurement is conducted concurrently to assess whether up-regulation of surface maker expression occurs at sub-cytotoxic concentrations. The prediction model use the relative fluorescence intensity of surface markers compared to solvent control to discriminate between sensitizers and non-sensitizers. Among the 166 substances tested in this *in vitro* assay, the allergenic drugs benzocaine, clofibrate, pyridine, hydroquinone, p-benzoquinone and diphenylclopropenone resulted correctly classified (Nukada et al., 2012; Takenouchi et al., 2013; Urbisch et al., 2015).

Overall, even if the number of drugs tested is very limited, data suggests that pharmaceuticals may share with chemical allergens a common mechanism of action that activates dendritic cells, and support the possibility to use these *in vitro* methods

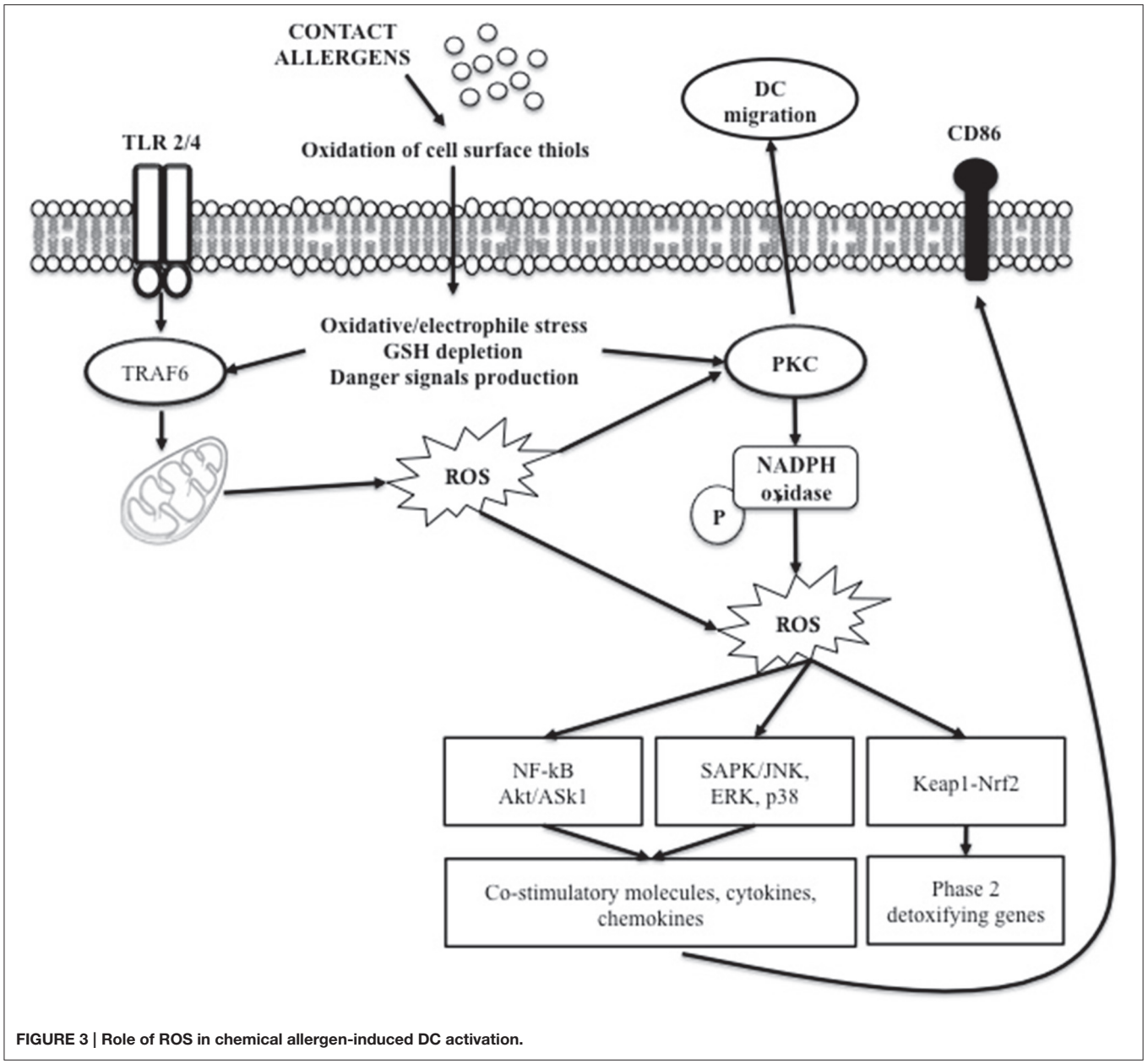

FIGURE 3 | Role of ROS in chemical allergen-induced DC activation.

also for the identification of drugs potentially associated with hypersensitivity reactions.

The THP-1 Activation Assay

As mentioned above, most drugs are small molecules and are by themselves, not immunogenic. During the haptenization process, these small molecules bind to carrier proteins to form a complete immunogenic complex (Chang and Gershwin, 2010). The hypersensitivity reaction then requires the activation and maturation of dendritic cells (DCs), which will then drive the activation of specific T cells (Martin, 2012). DCs are antigen-presenting cells (APC) that play a central role in the initiation and regulation of adaptive immune responses. Following the contact with antigens, DCs undergo a process of maturation associated with the expression of several co-stimulatory molecules on the membrane such as CD80, CD86 and CD40, various adhesion molecules (CD2, CD11a, CD54, CD58), and secrete different cytokines, including IL-1β and IL-8 (Quah and O'Neill, 2005). Once activated, DCs migrate into the regional lymph node or in the spleen, where they present antigen to specific T lymphocytes, through MHC class II molecules (Ryan et al., 2007) and co-stimulatory adhesion molecules expressed on both DC (i.e., CD86) and T cell (i.e., CD28) to ensure the necessary contact to achieve full T-cell activation. Following stimulation, a clone of T cells is produce with the ability to react to the antigen, resulting in the clinical manifestation of HDR.

Within the European project SENS-IT-IV, we have previously established an *in vitro* method able to identify contact and

respiratory allergens based on the use of the human THP-1 cell line (the same cell line used in the h-CLAT) and IL-8 release: the THP-1 activation assay (Mitjans et al., 2008, 2010). IL-8 is a potent chemotactic peptide for neutrophils as well as for T lymphocytes, basophils, and NK cells. In parallel to IL-8 production, several of the proposed *in vitro* methods, including the ones mentioned above, are based on DC and CD86 alone or in combination with CD54 expression for the identification of chemical sensitizers, due to their roles in antigen presentation and T cell activation.

Based on the notion that drug sensitizers and chemical sensitizers share the same mode of action, we recently investigated the possibility to use the THP-1 activation assay developed for skin and respiratory sensitizers, for the *in vitro* identification of pharmaceuticals, which may be associated with *in vivo* drug hypersensitivity reactions (Corti et al., 2015). It is well known that allergen drugs share with chemical allergens common mechanisms of cell activation and for reason we propose the THP-1 activation assay also for the hazard identification of immune-mediated hypersensitivity reactions induced by pharmaceuticals. Drugs were selected on the basis of clear *in vivo* immune-adverse reactions reported in literature, post-marketing data or labeling information, and on the commercial availability as pure drugs. Clonidine, ofloxacine, procainamide, streptozotocin, sulfamethoxazole which have been associated with a relatively high incidence of immune-mediated hypersensitivity reactions (Weaver et al., 2005), methyl salicylate and probenecid, which have been reported to cause irritant or allergic contact dermatitis and anaphylactic reactions (Corti et al., 2015), have been tested. We developed a strategy based on IL-8 production, CD86 and/or CD54 expression in THP-1 cells useful for the *in vitro* identification of drug sensitizers (see **Figure 2**). There are some important differences with the previous mentioned h-CLAT. First, the method we use to calculate CD86 and CD54 expression is different from h-CLAT protocol: in h-CLAT only the MFI is considered, while we also include the percentage of positive cells. The second point is the concentration tested. In fact, we observed that quite often a CV75 couldn't be reached with drugs compared to chemicals, meaning that drugs are less cytotoxic. The test we developed allowed the correct identification of all the selected drugs tested, including sulfamethoxazole, probenecid and procainamide for which metabolism is needed. Penicillin G, another drug frequently associated with hypersensitivity reactions (Siegel and Coleman, 1957), was previously tested, and found to be able to induced a dose-related release of IL-8 following 48 h of exposure (Mitjans et al., 2008).

Exposure of THP-1 cells to sensitizing drugs results in most cases in dose related release of IL-8 and increase in CD86 expression, with some differences among drugs, markers and times of exposure. As shown in **Table 1**, the combination of both IL-8 and CD86 expression allows the identification of all drugs tested. The use of IL-8 mRNA expression at 3 h or CD54 expression at 24 h may offer an alternative to the 48 h exposure and increase our confidence in the negativity of a drug (see Corti et al., 2015). The expression of IL-8 mRNA at 3 h is based on previous observation we made on chemical allergens failing to induce the release of IL-8: all chemicals sensitizers tested including pro-hapten induced IL-8 mRNA at 3 h (Galbiati et al., 2012).

Using streptozotocin as reference drug to study the mechanisms of action, we could demonstrate a key role for p38 mitogen-activated protein kinase (p38 MAPK) and PKC-β activation in streptozotocin-induced IL-8 release and CD86 expression (Corti et al., 2015), confirming previous results obtained with chemical allergens (Mitjans et al., 2008; Corsini et al., 2014).

Evidence indicates that oxidative stress is involved in chemical-induced skin allergic and inflammatory diseases (Okayama, 2005; Byamba et al., 2010). Chemical-induced oxidation of the cell surface thiols appears to be one of the triggers of DC maturation, resulting in intracellular redox imbalance that generate stress-related signal (**Figure 3**). The Keap1/Nrf2-signaling pathway is dedicated to the detection of electrophilic stress in cells leading to the up-regulation of genes involved in protection or neutralization of chemicals reactive species (Natsch and Emter, 2007). It has been shown in human monocyte-derived dendritic cells that chemical sensitizers induced oxidative stress measured by the glutathione GSH/GSSG ratio, as a redox marker (Mizuashi et al., 2005). The reduction of the glutathione GSH/GSSG ratio was accompanied by CD86 up-regulation and p38 MAPK activation, suggesting that the electrophilic properties of chemicals sensitizers may be perceived by DCs as a danger signal leading to DC maturation (Sasaki and Aiba, 2007). Engagement of certain Toll like receptors (TLR1, 2, and 4) leads to mitochondrial translocation of the signal adaptor TRAF6. At the mitochondria, TRAF6 interacts with ECSIT, a protein implicated in the assembly of complex I, leading to its ubiquitylation, which results in increased ROS production.

TABLE 2 | Drugs known to induce hypersensitivity and resulted positive in DC-based *in vitro* methods.

Drug	h-CLAT	MUSST	mMUSST	THP-1 activation assay
Benzocaine	*	#		
Clofibrate	x			
Pyridine	*			
Hydroquinone	*	#		
p-Benzoquinone	*	#	+	
Diphenylclopropenone	*	#		
Streptozotocin				§
Sulfamethoxazole				§
Procainamide				§
Ofloxacin				§
Neomycin				§
Clonidine				§
Methyl salicylate				§
Probenecid				§
Metformin				§

*, *Nukada et al. (2012)*; x, *Takenouchi et al. (2013)*; #, *Natsch et al. (2013)*; +, *Bauch et al. (2012)*; §, *Corti et al. (2015)*.

Proteins that are reversibly modulated by ROS are of high interest. In this context, protein kinases and phosphatases, which act co-ordinately in the regulation of signal transduction through the phosphorylation and dephosphorylation of target proteins, have been described to be key elements in ROS-mediated signaling events. In particular, PKC isoforms have been shown to contain a unique structural feature that is susceptible to oxidative modification (Cosentino-Gomes et al., 2012). The high levels of cysteine residues render the regulatory domain susceptible to redox regulation (Gopalakrishna and Jaken, 2000; Giorgi et al., 2010). Currently, evidence supports the direct activation of different PKC isoforms by ROS generation; in particular the β isoform is able to induce ROS generation through mitochondrial damage (Pinton et al., 2007).

To summarize the drugs tested in the preclinical available *in vitro* methods and known to be able to induce hypersensitivity reactions, a list is reported in **Table 2**.

CONCLUSION

Develop a new pharmaceutical entity has many possibilities of failure and is a very expensive process. Methods and/or models to assess the hazard of hypersensitivity reactions by pharmaceuticals are not yet validated (or requested on a routine basis). The assessment of hypersensitivity of pharmaceuticals would benefit from a rationalistic approach using clear and linear test methods that are based on immunological knowledge and read out parameters. There are many *in vitro* tests that can help in diagnosis and identification of the pharmaceuticals able to cause a hypersensitivity reaction, and among of these, we established, using the THP-1 cell line as surrogate of dendritic cells, experimental conditions and markers to correctly identify drug sensitizers. The THP-1 cells assay can be easily incorporated into drug development to identify drugs that may have the potential to cause systemic hypersensitivity reactions.

AUTHOR CONTRIBUTIONS

VG wrote the manuscript; AP, and EK performed the experiments depicted in **Figure 3**; EC supervised the overall project and finalized the final version of the review. All authors have read this review and gave their agreement for submission.

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Basophil Reactivity as Biomarker in Immediate Drug Hypersensitivity Reactions—Potential and Limitations

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Immediate drug hypersensitivity reactions (DHRs) resemble typical immunoglobulin E (IgE)-mediated symptoms. Clinical manifestations range from local skin reactions, gastrointestinal and/or respiratory symptoms to severe systemic involvement with potential fatal outcome. Depending on the substance group of the eliciting drug the correct diagnosis is a major challenge. Skin testing and *in vitro* diagnostics are often unreliable and not reproducible. The involvement of drug-specific IgE is questionable in many cases. The culprit substance (parent drug or metabolite) and potential cross-reacting compounds are difficult to identify, patient history and drug provocation testing often remain the only means for diagnosis. Hence, several groups proposed basophil activation test (BAT) for the diagnosis of immediate DHRs as basophils are well-known effector cells in allergic reactions. However, the usefulness of BAT in immediate DHRs is highly variable and dependent on the drug itself plus its capacity to spontaneously conjugate to serum proteins. Stimulation with pure solutions of the parent drug or metabolites thereof vs. drug-protein conjugates may influence sensitivity and specificity of the test. We thus, reviewed the available literature about the use of BAT for diagnosing immediate DHRs against drug classes such as antibiotics, radio contrast media, neuromuscular blocking agents, non-steroidal anti-inflammatory drugs, and biologicals. Influencing factors like the selection of stimulants or of the identification and activation markers, the stimulation protocol, gating strategies, and cut-off definition are addressed in this overview on BAT performance. The overall aim is to evaluate the suitability of BAT as biomarker for the diagnosis of immediate drug-induced hypersensitivity reactions.

Keywords: antibiotics, basophil activation test, biologicals, chemotherapeutics, fluoroquinolones, NMBAs, NSAIDs, RCM

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KEY PLAYERS IN THE IMMEDIATE-TYPE ALLERGIC EFFECTOR PHASE

Key characteristic of allergic effector cells in immediate-type allergy is allergen-specific IgE bound to the high affinity IgE receptor, i.e., FcεRI, on the cell surface. Capturing of allergens by surface IgE results in FcεRI crosslinking and elicits the acute phase of the allergic response involving the sudden release of vasoactive mediators into the tissue and/or circulation. This sudden activation process and mediator release is termed anaphylactic degranulation (depicted in **Figure 1A**) and

may induce life-threatening anaphylaxis (Hoffmann, 2015). Mast cells and basophils both share these key characteristics. Mast cells reside in the tissue and are considered primary allergic effector cells. Basophils are peripheral blood granulocytes, easily accessible via venipuncture and a well-established surrogate for allergy diagnosis (MacGlashan, 2013).

THE BASOPHIL ACTIVATION TEST

Basophil activation can be measured by flow cytometry and multicolor staining with fluorescent-labeled detection antibodies targeting specific identification and activation markers on the surface of basophils. The most common identification strategies use surface IgE, eotaxin CC chemokine receptor 3 (CCR3), the combination of interleukin 3 receptor alpha chain $CD123^{high}$ with human leukocyte antigen $HLA\text{-}DR^{neg}$, the combination of prostaglandin D_2 receptor $CRTH\text{-}2^{high}$ with $CD3^{neg}$, or the basophil-specific ectonuclease CD203c. **Table 1** provides an overview of basophil identification strategies in drug hypersensitivity research which we numbered "strategy 1" through "strategy 6" according to the frequency they have been used. For basophil activation the degranulation marker lysosomal-associated membrane glycoprotein-3 (LAMP-3), also termed CD63, or upregulation of CD203c are determined. Thus, changes in activation state can be quantified on a single cell basis. Degranulation means fusion of specific intracellular vesicles filled with preformed mediators, the so-called granules, with the plasma membrane and a transition of CD63 from inside out. The result is a sudden and pronounced rise, i.e., log-shift, of the fluorescence intensity signal, in the detection of surface CD63. Concomitantly, upregulation of CD203c has been observed which can be detected as significant increase in the mean fluorescence intensity signal of the CD203c detection antibody. Flow cytometry dot plots for both activation scenarios are depicted in **Figure 1B**. For further details about assay parameters of BAT the interested reader is referred to the comprehensive review of McGowan and Saini (2013).

In addition to anaphylactic degranulation another type of basophil activation termed piecemeal degranulation has been described (Dvorak, 2005). This alternative activation mechanism may also lead to altered surface expression of activation markers which can be assessed by BAT (Hausmann et al., 2009; MacGlashan, 2010). Consequently, BAT has been recognized as a promising tool for *in vitro* diagnosis of allergy or other hypersensitivity reactions including immediate adverse reactions to various drugs (Hoffmann et al., 2015).

TECHNICAL ISSUES OF BASOPHIL ACTIVATION TESTING

Usually BAT is performed from either heparinized, citrate- or EDTA-anticoagulated whole blood collected from allergic/hypersensitive donors (**Table 1**). When EDTA is used as anticoagulant Ca^{++} has to be supplemented to enable proper degranulation. For *in vitro* stimulation of basophils the samples are incubated with the allergen/drug or buffer only (negative control) for several minutes to hours at 37°C. As positive control, anti-IgE antibodies, anti-FcεRI antibodies, and formyl-methionine-leucine-phenylalanine (fMLF) are used. Latter represents an alternative degranulation/activation stimulus and is important to demonstrate basophil functionality in case of donors whose basophils fail to react *in vitro* to IgE-mediated pathway stimulation, so-called non-responders (Eberlein et al., 2010; MacGlashan, 2013).

Next, basophil identification and activation markers are stained with fluorescently labeled antibodies, subsequently erythrocytes are lysed. Depending on the protocol, staining can be performed during basophil stimulation in a single step. Upon flow cytometric acquisition of at least 200, in the optimal case 500–1000 basophils, activation marker expression is compared between buffer-treated samples and allergen-/drug-stimulated basophils. Different evaluation strategies are used. Some studies set the cut-off for spontaneously activated basophils arbitrarily at 5%, whereas others use stimulation indices of %CD63-/CD203c-positive cells, i.e., SI(%), or mean fluorescence intensities (MFI) of activation markers, i.e., SI, compared to negative control (**Table 1**). For interpretation of BAT area under the dose curve (AUC) measurements have recently been postulated. These enable a combined evaluation of basophil reactivity, i.e., the dose (range) at which maximal response occurs, and basophil sensitivity, i.e., the dose at which half of the maximal response occurs. As the AUC representation incorporates partial energy, which may arise at high allergen concentrations, and can be calculated even in cases where responses do not fit the typical shape of dose–response curves, it is particularly useful for monitoring the efficacy in allergen-specific immunotherapy (Ebo et al., 2004; Hausmann et al., 2009; Hoffmann et al., 2015).

BASOPHIL ACTIVATION TEST WITH DRUGS—BACKGROUND CONSIDERATIONS

Small molecular weight drugs constitute haptens which are not capable of FcεRI crosslinking themselves (hapten concept; Pichler et al., 2011). They require conjugation to carrier molecules (**Figure 1A**), usually abundant blood proteins, for eliciting an immune reaction in susceptible individuals. Moreover, reactive intermediates may be formed by drug metabolism (pro-hapten concept; Park et al., 1998; Naisbitt et al., 2000). Therefore, the use of drug metabolites and hapten-carrier conjugates has been promoted for the investigation of drug hypersensitivity reactions (Himly et al., 2003; Harrer et al., 2010; Steiner et al., 2011, 2014). Of note, in a case of propyphenazone (PP) hypersensitivity basophils reacted in BAT solely upon stimulation with the drug-carrier conjugate but not with pure PP (Steiner et al., 2014). Nevertheless, BAT is most frequently performed with solutions of plain drugs, a consequence of lacking knowledge in regard to relevant determinants, metabolic intermediates, their reactive functions, required linker length to the carrier molecule, and hapten orientation.

Alternative to the hapten and pro-hapten concepts in DHRs, the p-i concept has become well-accepted, however, it primarily

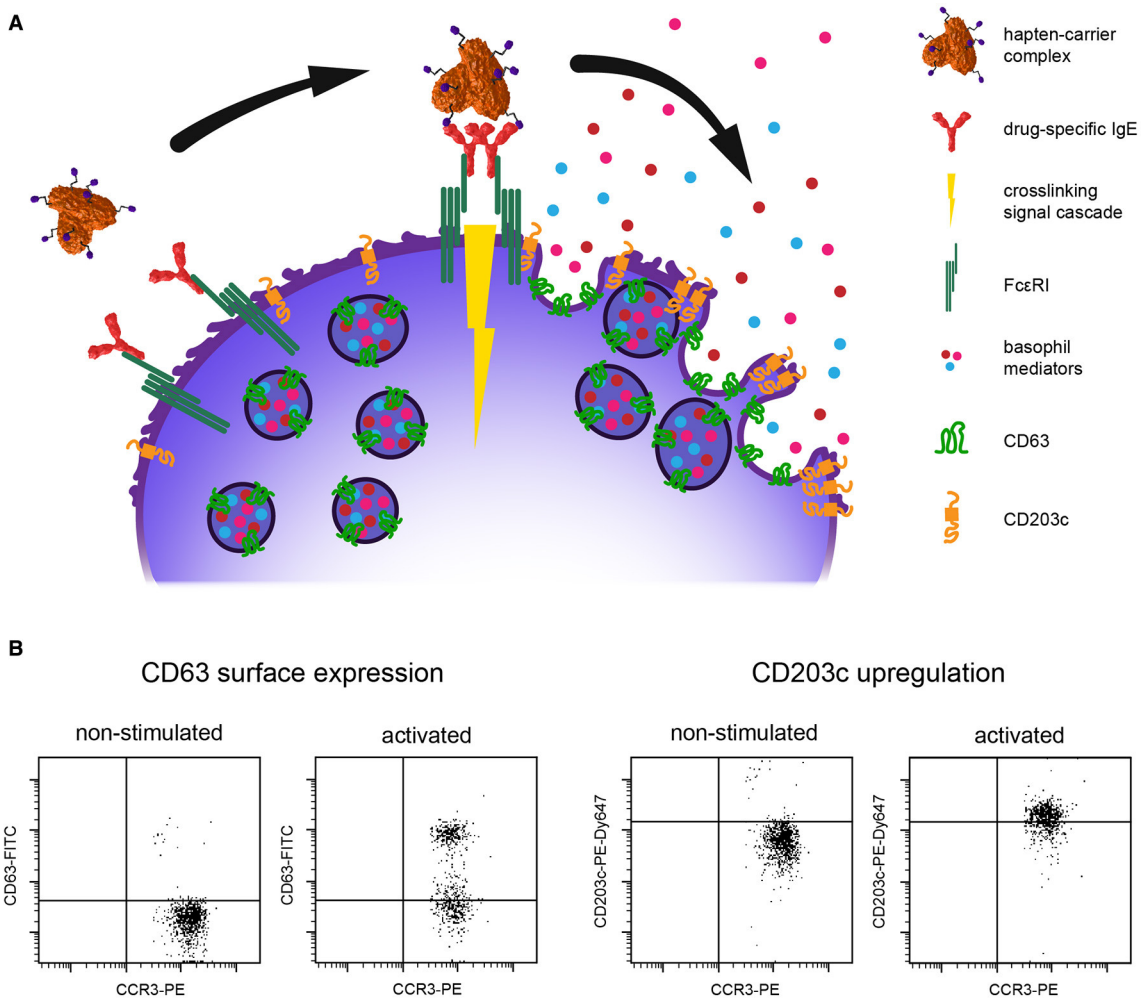

FIGURE 1 | (A) Schematic illustration of IgE-mediated cross-linking. Upon binding of the hapten-carrier complex to the drug-specific IgE captured on high affinity IgE receptor FcεRI, basophils react by degranulation and mediator release. CD63 is transferred from the vesicle to the plasma membrane and CD203c is upregulated, thus both serve as activation markers. **(B)** Flow cytometry dot plots of typical activation patterns for CD63 and CD203c; CCR3, basophil identification marker; PE, phycoerthrin label; FITC, fluorescein label; PE-Dy647, tandem label.

accounts for T cell-mediated delayed-type immune reactions against drugs such as lidocaine, sulfamethoxazole, lamotrigine, carbamazepine, p-phenylendiamine, etc. or against metals like in nickel contact dermatitis (Pichler et al., 2006). DHRs of this kind are elicited on a different time-scale, not discussed here, and an involvement of basophils is unlikely. A comprehensive overview can be gained from reviews by Pichler et al. (Pichler, 2003; Pichler et al., 2015).

BAT FOR THE EVALUATION OF IMMEDIATE DRUG HYPERSENSITIVITY TO DIFFERENT DRUG CLASSES

In the following paragraphs the suitability of basophil activation as a biomarker for evaluating immediate hypersensitivity reactions to different drug classes is discussed. The assay parameters used and activation patterns observed in the cited studies are summarized in **Table 1**.

Basophil Activation in Antibiotics or Quinolone Hypersensitivity

Immediate DHRs against beta-lactam antibiotics such as penicillin, amoxicillin, and cephalosporin have been broadly investigated and are most likely IgE-mediated. Diagnosis of beta-lactam allergy first place is based on skin prick and intradermal tests. Sensitivity of skin tests, however, does not exceed 50–70%. The *in vitro* diagnostic method of quantifying beta-lactam-specific IgE antibodies (Mangodt et al., 2015) is an important complementary information. Clinically validated tests for drug-specific IgE, however, are difficult to develop, require complex coupling reactions for attaching the drug hapten onto a solid phase for antibody recognition, and are available only for a limited number of antibiotics. Instead, simple drug dilutions

are used in BAT. It thus, appears a promising tool for *in vitro* diagnosis of beta-lactam allergy. Several groups investigated the applicability of BAT in the diagnostic management of beta-lactam allergy (Sanz et al., 2002; Gamboa et al., 2004a; Torres et al., 2004, 2010a, 2011; Abuaf et al., 2008; De Week et al., 2009; Eberlein et al., 2010; Garcia-Ortega and Marin, 2010). BAT performance, however, varied between groups with a median sensitivity of 50% (range 22–55%) and specificity ranging from 79 to 100%. Quintessence of these studies is that BAT was superior to immunoassaying for drug-specific IgE, but not to skin testing. Importantly, skin testing and BAT do not necessarily corroborate each other. Positive skin testing has been confirmed by BAT only in about 50–60% of patients (De Week et al., 2009; Torres et al., 2010a), whereas up to one third of skin test-negative patients have been identified by BAT (De Week et al., 2009). The tenor across studies thus was that the role of BAT currently is complementary, respectively supplementary, in the diagnosis of beta–lactam allergy.

Factors possibly contributing to the observed variance in sensitivity of BAT may involve patients selection criteria such as severity of reactions and time elapsed since the reaction (optimum: 1–6 months), regional preferences in drug prescriptions, and whether the drugs tested allow identification of cross-reactors. Methodological variations such as differential activation times (range 20–40 min) and different activation markers (CD63 and/or CD203c) additionally complicate comparability of results.

One key question is why should basophils degranulate in an IgE-dependent fashion *in vitro* upon stimulation with a dilution of monomeric small antibiotic haptens? One theory is that the beta-lactam ring confers instability to the compound facilitating the conjugation of the drug to abundant blood proteins such as albumin, transferrin and/or immunoglobulins (Torres et al., 2016). Once attached to a carrier protein the side chain of the drug, the thiazolidine ring, or the conjugation site itself are immunogenic in susceptible individuals and capable of both, eliciting an IgE response and crosslinking of surface-bound IgE.

Beyond diagnostic purposes one important application of BAT is determining an IgE-mediated pathomechanism when drug-specific IgE cannot be evidenced. Wortmannin, for instance, is a strong inhibitor of phosphatidylinositol 3-kinase (PI3K) and inhibits basophil activation in response to FcεRI crosslinking but not to stimulation with fMLF. In patients with selective allergy to the beta-lactamase inhibitor clavulanic acid, presence of drug-specific IgE was suspected but not detected. Using BAT an IgE-mediated pathomechanism was confirmed as BAT became negative upon stimulation with clavulanic acid in presence of wortmannin (Torres et al., 2010b).

BAT may be useful also in the diagnosis of immediate hypersensitivity reactions to quinolones. Diagnosis of quinolone allergy mainly relies on patient history and clinical manifestation. Skin tests are hampered by false positive reactions due to skin irritation rendering the positive predictive value of skin testing close to chance results. Drug-specific IgE have been reported, however, validated assays do not exist (Manfredi et al., 2004; Aranda et al., 2011; Mayorga et al., 2013). Aranda et al. showed both drug-specific IgE and dose-responsiveness in BAT for ciprofloxacin, moxifloxacin and levofloxacin, and inhibition of BAT positivity with wortmannin (Aranda et al., 2011). Moreover, they reported a higher sensitivity of BAT compared to IgE testing. Two out of seven studies evaluating BAT in quinolone hypersensitivity were negative with 0% sensitivity (Seitz et al., 2009; Lobera et al., 2010). The patient collectives of these two studies were quite small though as only 6, respectively, 4 patients were included. Contrary, Rouzaire et al. (2012) tested 34 patients and reported a very good negative predictive value of BAT as quinolones were successfully reintroduced in 15 of the 17 patients (50%) who tested negative in BAT. They emphasized the importance of negative BAT results in quinolone hypersensitivity as criteria for provocation test and thus the opportunity to possibly and safely reintroduce the drug (Rouzaire et al., 2012). Photodegradation could be one cause for false negative results. This was shown for moxifloxacin as positive results doubled when BAT was performed in the dark (Mayorga et al., 2013). Taken together, BAT appears helpful in the management of fluoroquinolone hypersensitivity.

Basophil Activation in Neuromuscular Blocking Agent Hypersensitivity

Anaphylactic episodes during general anesthesia have severe implications for the patient. Neuromuscular blocking agents (NMBAs) including rocuronium, vecuronium, atracurium, cisatracurium, and suxamethonium account for >60% of such cases with a high degree of cross-reactivity within this drug group and even further to opioid antitussives such as codeine or morphine, with the existence of drug-specific IgE demonstrated (Baldo and Fisher, 1983; Vervloet et al., 1983; Sainte-Laudy et al., 2006; Ebo et al., 2007; Leysen et al., 2013). However, it has been recognized that IgE accounts for immediate DHRs against NMBAs only in ∼50% of cases. Therefore, a number of studies evaluated BAT protocols aiming at a more reliable before-hand screening tool (Monneret et al., 2000, 2002; Sudheer et al., 2005; Ebo et al., 2006; Kvedariene et al., 2006; Sudheer and Appadurai, 2007). Sudheer et al. (2005) compared CD63, CD203c, histamine release and skin testing for their predictive values. While specificities reached 100%, the sensitivities of these four techniques determined for the whole NMBA group in their cohort of 21 patients resulted in 79, 36, 36, and 64%, respectively. Other studies reported specificities for BAT based on CD63 expression of >93% with sensitivities of >54%. As CD63 enables a better judgment of the type of basophil activation than CD203c, i.e., creating a log-shift in the flow cytometric dot plots, Ebo et al. (2006) were able to reach a sensitivity for DHR against rocuronium >91.7% using 0.5 mg/ml of drug and setting the diagnostic threshold value at 4% to be most discriminative, as they determined from two-graph receiver operating characteristics. In a large study involving thorough diagnostic workup of 104 patients the same group (Leysen et al., 2011) obtained a positive predictive value of 98% for rocuronium using the combination of skin testing, BAT, and drug-specific IgE testing by ImmunoCAP. In their study, skin tests turned out most reliable, however, when these are negative, BAT is indicated. For assessment of cross-reactivity between rocuronium and

TABLE 1 | Overview on BAT applications for different drug classes, identification markers, acquisition conditions, observed activation patterns, and verification of IgE.

References	Drugs	Drug-specific serum IgE	Anti-coagulant	Basophil activation test protocols				
				Basophil identification strategies	Stimulation conditions	Acquisition details		
					Time [min] at 37°C	Activation marker	Basophils (n)	Cut-off
1. BETA-LACTAM ANTIBIOTICS								
Eberlein et al., 2010	PCN G, PCN V, PPL, MDM, AMP, AX, CFX	Detected	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	15	CD63	300	≥5%, SI ≥ 2
Abuaf et al., 2008	AX, AMP, CFX,	Not detected	HEP	Strategy 4^{+}: SSC^{low}, $CD33^{dim}$, $CD45^{pos}$, IgE^{pos}	30	CD63, CD203c	200	Mean n.c.+ 2 × *SD*
De Week et al., 2009	PPL, MDM, BPN, AX, AMP, CEF	Detected	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Garcia-Ortega and Marin, 2010	AX	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	10^{*}+20	CD63	1000	≥5%, SI ≥ 2
Sanz et al., 2002	BP, AMP, AX, MDM, PPL	Detected	ACD	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Torres et al., 2010a	AX, DKP	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	10^{*}+20	CD63	1000	SI(%)>2
Torres et al., 2004	AX, BP, BPP, AMP, MDM	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	10^{*}+20	CD63	1000	SI(%)>2
Torres et al., 2011	AX	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	10^{*}+20	CD63	1000	SI(%)>2
2. FLUOROQUINOLONES								
Aranda et al., 2011	CPX, LVX, MOX	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	250–500	≥5%, SI ≥ 2
Mayorga et al., 2013	CPX, MOX	Not detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	250–500	≥5%, SI ≥ 2
Lobera et al., 2010	CPX, LVX, MOX, NFX	Not detected	HEP	Strategy 2: SSC^{low}, $CD123^{pos}$, $HLA\text{-}DR^{neg}$	15	CD63	200	≥5%, SI ≥ 2
Seitz et al., 2009	CPX, OFX, LVX, MOX	Not detected	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	n.a.	CD63	n.a.	n.a.
Rouzaire et al., 2012	LVX, OFX, CPX, MOX, LMX, NFX, FLU, PPA	Not detected	HEP	Strategy 1^{+}: SSC^{low}, IgE^{pos}, $CD45^{pos}$	10	CD203c	n.a.	>10%
Blanca-Lopez et al., 2013	NFX, CPX, LVX, MOX	n.a	HEP	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	250–500	≥5%, SI ≥ 2
Ben Said et al., 2010b	CPX, NFX, OFX	Not detected	n.a.	n.a.	n.a.	CD203c	n.a.	n.a.
3. NEURO-MUSCULAR BLOCKING AGENTS (NMBAs)								
Ebo et al., 2007	ROC, SUX, MSO4, PHO	Detected	HEP	n.a.	n.a.	n.a.	n.a.	n.a.
Leysen et al., 2013	ROC, VEC, ATR, cATR, SUX	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	20	CD63, CD203c	n.a.	n.a.
Sainte-Laudy et al., 2006	ROC, VEC, cATR, SUX	Detected	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	500	SI > 2
Ebo et al., 2006	ROC	Not detected	HEP	Strategy 2: SSC^{low}, $CD123^{pos}$, $HLA\text{-}DR^{neg}$	20	CD63	500	n.a.
Kvedariene et al., 2006	ROC, VEC, ATR, PANC, SUX	n.a	HEP	Strategy 1: SSC^{low}, IgE^{pos}	n.a.	CD63	1000	>15%
Monneret et al., 2002	ROC, VEC, ATR, MIV, SUX	Detected	n.a.	Strategy 3^{++}: SSC^{low}, $CCR3^{pos}$, IgE^{pos}, $CD45^{pos}$	10	CD63	n.a.	>2%
Monneret et al., 2000	ROC, SUX, PROP, MDL, ALF, THI	Detected	n.a.	n.a.	n.a.	CD63	n.a.	n.a.

(Continued)

TABLE 1 | Continued

References	Drugs	Drug-specific serum IgE	Anti-coagulant	Basophil activation test protocols				
				Basophil identification strategies	Stimulation conditions	Acquisition details		
					Time [min] at 37°C	Activation marker	Basophils (n)	Cut-off
Sudheer and Appadurai, 2007	VEC, cATR, SUC	Not detected	HEP	n.a.	n.a.	CD63	n.a.	n.a.
Sudheer et al., 2005	ROC, VEC, ATR, ALC, SUX	Not detected	HEP	Strategy 1^{+}: SSC^{low}, IgE^{pos}, $CD45^{pos}$	15	CD63, CD203c	n.a.	>10%
Leysen et al., 2011	ROC, VEC	Detected	HEP	Strategy 2: SSC^{low}, $CD123^{pos}$, $HLA\text{-}DR^{neg}$	n.a.	CD63	n.a.	>4%
4. RADIO CONTRAST MEDIA (RCM)								
Bohm et al., 2011	IOT, IOP	n.a	HEP	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	n.a	>5%
Pinnobphun et al., 2011	IOP, PM, IOB, IOH, IOX	n.a	EDTA	Strategy 3^{+}: SSC^{low}, $CCR3^{pos}$, IgE^{pos}	n.a	CD63	500	
Salas et al., 2013	IOH, IOD, IOM, IOB	Detected	n.a.	Strategy 1: SSC^{low}, IgE^{pos}	10^{*}+20	CD63	1000	SI(%) > 2
Trcka et al., 2008	IOP, IOM, IPN	n.a	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	n.a	CD63	500	>5%
5. PLATIN CHEMOTHERAPEUTICS								
Iwamoto et al., 2014	CPT	detected	EDTA	n.a	30	CD203c	n.a	>2%
Iwamoto et al., 2012	CPT	n.a	EDTA	Strategy 5: SSC^{low}, CRTH-2^{pos}, $CD3^{neg}$	30	CD203c	n.a	>2%
6. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)								
Harrer et al., 2010	DF	Not detected	EDTA	Strategy 3: SSC^{low}, $CCR3^{pos}$	20	CD63	500–1000	≥%, SI ≥ 2
Steiner et al., 2011	PP	Detected	EDTA	Strategy 3: SSC^{low}, $CCR3^{pos}$	45	CD63	500	≥5%
Abuaf et al., 2012	ASA, ACET, KET, DF, CEL	n.a	HEP	Strategy 4: SSC^{low}, $CD33^{dim}$, $CD45^{pos}$	30	CD63, CD203c	400	Mean n.c. + 2 × *SD*
Bavbek et al., 2009	ASA, DF	n.a	ACD	Strategy 5: SSC^{low}, CRTH-2^{pos}, $CD3^{neg}$	40, 15	CD63, CD203c	n.a.	≥5%, SI > 2
Celik et al., 2009	ASA	n.a	EDTA	Strategy 6: SSC^{low}, $FceRI^{pos}$	180	CD63, CD203c	500	MFI, SI > 2
Gamboa et al., 2004b	ASA, ACET, MET, DF, NAP	n.a	ACD	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Gamboa et al., 2003a	MET	n.a	ACD	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Gomez et al., 2009	MET	n.a	HEP	Strategy 1: SSC^{low}, IgE^{pos}	30	CD63	250-500	≥5%, SI > 2
Hagau et al., 2013	DIP	n.a	EDTA	Strategy 3: SSC^{low}, $CCR3^{pos}$	15	CD63	500	≥5%, SI ≥ 1.71
Kim and Cho, 2012	ASA, IBU, DF	n.a	EDTA	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	n.a.	≥5%, SI ≥ 2
Korosec et al., 2011	ASA	n.a	HEP	Strategy 2: SSC^{low}, $CD123^{pos}$, $HLA\text{-}DR^{neg}$	15	CD63	500	% CD63 positive
Malbran et al., 2007	DF	n.a	HEP	Strategy 1^{+}: SSC^{low}, IgE^{pos}, $CD45^{pos}$	15	CD63	200	Mean n.c. + 2 × *SD*

(Continued)

TABLE 1 | Continued

References	Drugs	Drug-specific serum IgE	Anti-coagulant	Basophil activation test protocols				
				Basophil identification strategies	Stimulation conditions	Acquisition details		
					Time [min] at 37°C	Activation marker	Basophils (n)	Cut-off
Sanz et al., 2005	ASA, ACET, MET, DF, NAP	n.a	ACD	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Phillips-Angles et al., 2016	ETO, MET	Clinically excluded	HEP	Strategy 2: SSC^{low}, $CD123^{pos}$, $HLA\text{-}DR^{neg}$	15	CD63	500	≥5%, SI ≥ 2
7. OTHER DRUGS								
Soriano Gomis et al., 2012	GA	Detected	n.a.	Strategy 1: SSC^{low}, IgE^{pos}	40	CD63	500	≥5%, SI ≥ 2
Aranda et al., 2010	MPS	Detected	HEP	Strategy 1: SSC^{low}, IgE^{pos}	n.a	CD63	1000	SI ≥ 2
Ben Said et al., 2010a	MPS	n.a.	n.a.	n.a.	n.a	CD203c	n.a	>2%
Gamboa et al., 2003b	OPZ	n.a	ACD	n.a.	n.a	CD63	n.a	SI
Apostolou et al., 2006	GF	n.a	HEP	Strategy 1: SSC^{low}, IgE^{pos}	20	CD63	n.a	>6%

*1. Beta-lactam antibiotics: amoxicillin (AX), ampicillin (AMP), benzylpenicillin (BP), benzylpenicilloyl-PLL (BPN-PLL), cefuroxim (CFX), cephalosporins (CEPH), diketopiperazine (DKP), minor determinant mixture (MDM), penicillin G (PCN G), penicillin V (PCN V), penicilloyl polylysine (PPL); 2. Fluoroquinolones: ciprofloxacin (CPX), flumequin (FLU), levofloxacin (LVX), lomefloxacin (LMX), moxifloxacin (MOX), norfloxacin (NFX), ofloxacin (OFX), pipemidic acid (PPA); 3. NMBAs: alcuronium (ALC), alfentanyl (ALF), atracurium (ATR), cisatracurium (cATR), midazolam (MDL), mivacurium (MIV), morphine (MSO4), pancuronium (PANC), pholcodine (PHO), propofol (PROP), rocuronium (ROC), succinylcholine (SUC), suxamethonium (SUX), thiopental (THI), vecuronium (VEC); 4. RCM: iobitridiol (IBT), iodixanol (IDX), iohexol (IOH), iomeprol (IOM), iopamidol (IPM), iopentol (IPN), iopromide (IOP), iotrolan (IOT), ioxithalamate (IOX); 5. Chemotherapeutics: carboplatin (CPT), cisplatin (CsPT); 6. NSAIDs: acetaminophen (ACET), acetyl salicylic acid (ASA), celecoxib (CEL), diclofenac (DF), dipyrone (DIP), etoricoxib (ETO), ibuprofen (IBU), ketoprofen (KET), metamizol (MET), naproxen (NAP), propyphenazon (PP); 7. Other drugs: gelofusine (GF), glatiramer acetate (GA), methyolprednisolone (MPS), omeprazol (OPZ); ethylenediaminetetraacetic acid (EDTA), heparin (HEP), acid citrate dextrose (ACD); n.a., not available; SSC, side scatter; MFI, mean fluorescence intensity; n.c., negative control; n, number; SD, standard deviation; SI, stimulation index of MFI activated compared to n.c.; SI(%), stimulation index of %CD63-/CD203c-positive compared to n.c.; +/++ indicate use of one/two additional identification marker(s) on top of the respective gating strategy; *, pre-stimulation in incubation buffer.*

vecuronium, the two most often used NMBAs, BAT was found to complement skin tests well.

Basophil Activation in Radiocontrast Media Hypersensitivity

Immediate DHRs against non-ionic radiocontrast media (RCM) occur in 0.7–3.1% of patients, with ionic RCM being even more prevalent, i.e., up to 12.7% including mild reactions or reactions due to rapid intravenous infusion of these highly osmolar substances (Stellato et al., 1996). Nevertheless, 0.04% of patients experience severe reactions upon administration of non-ionic RCM (Wolf et al., 1989; Katayama et al., 1990; Lieberman and Seigle, 1999). Traditionally, RCM reactions have been considered as non-IgE-mediated, and the majority of DHRs indeed seem to result from RCM non-specifically binding to surface receptors or indirectly interfering with the complement or kinin cascades, but skin testing and BAT have been successfully applied more recently (Brockow et al., 2009; Javaloyes et al., 2012; Philipse et al., 2013). Still few anecdotal reports on RCM-specific IgE exist, therefore, some studies have sought to differentiate IgE- from non-IgE-mediated mechanisms, however, have not determined the presence of drug-specific IgE themselves. Moreover, a number of studies have evaluated BAT as a diagnostic tool for RCM hypersensitivity, however, giving variable experimental details, which makes it difficult to judge the mode of basophil activation (Trcka et al., 2008; Bohm et al., 2011; Pinnobphun et al., 2011; Salas et al., 2013). Presence of drug-specific IgE was not shown in any of the reports. Some studies demonstrated log-shift activation of CD63 (Pinnobphun et al., 2011), while others did not (Bohm et al., 2011). Meta-analyzing the presented flow cytometric data some degree of inconsistency in the basophil activation profiles seems to be a common characteristic of immediate DHRs against RCM. Other influences may account as listed in a commentary by Chirumbolo (2013). For instance, a dose-dependent enhancement of CD63 expression upon pre-stimulation with interleukin 1β was observed by Boehm et al., however, the authors also reported variation of this effect between different RCM. Summarizing, one can say literature has demonstrated sensitivities of 46–63% and specificities of 89–100% for BAT qualified on the combination of skin and provocation testing serving as the current diagnostic standard for RCM hypersensitivity. In general, good correlation between skin test, drug provocation test, and BAT has proven useful to complement *in vivo* testing (Pinnobphun et al., 2011; Salas et al., 2013). Nevertheless, only in rare cases drug-specific IgE is involved.

Basophil Activation in Platinum-Containing Chemotherapeutic Hypersensitivity

The DHRs against carboplatin or cisplatin, compounds used repeatedly at high doses in anti-cancer therapy, include hyper- or hypotension, vomiting, dyspnea, and wheezing. They arise in up to 26.7% of patients and skin testing has been successfully performed although safety concerns exist regarding severe side-effects for the patient and risk of exposure of the medical personnel (Markman et al., 1999; Leguy-Seguin et al., 2007; Sugimoto et al., 2011). Consequently, the potential of BAT for predicting DHRs has been investigated using flow cytometric determination of CD203c (Iwamoto et al., 2012, 2014). These authors verified the involvement of IgE in carboplatin-induced DHR, as the basophil activation could be inhibited by wortmannin and anti-IgE pretreatment by omalizumab. In a case study of a severely anaphylactic history, BAT was applied evaluating the potential additive histamine-liberating effect by cisplatin following RCM administration (Viardot-Helmer et al., 2008). In conclusion, a similar situation to RCM seems to occur with platinum-containing anti-neoplastic drugs. In a low number of patients drug-specific IgE seems to be involved, still BAT may be suitable for diagnosis of severe immediate DHRs even if pharmacologic, i.e., non-immune-mediated mechanisms are underlying.

Basophil Activation in Analgesic Hypersensitivity

Non-steroidal anti-inflammatory drugs (NSAIDs) are known to frequently cause drug hypersensitivity reactions ranging from mild symptoms, e.g., skin reactions, to severe life-threatening systemic complications (Kowalski et al., 2011). Although the symptoms can mimic typical IgE-mediated reactions, drug-specific IgE seems not to be involved in most NSAID hypersensitivities, as NSAID-specific IgE has only been detected for PP (Himly et al., 2003). Investigating severe selective diclofenac (DF) hypersensitivity using drug-human serum albumin conjugates of DF and the most common DF metabolites no drug-specific IgE was detectable by ELISA (Harrer et al., 2010). Instead of an IgE-mediated mechanism underlying immediate DHRs to DF, it has been hypothesized that the inhibition of cyclooxygenase-1, triggering the reduction of prostaglandin E2 accompanied by an increase in leukotriene production, accounts for the observed type I-like symptomatic (Mastalerz et al., 2004). Regardless the lack of NSAID-specific IgE, BAT has been explored by several groups leading to conflicting results (Gamboa et al., 2003a, 2004b; Celik et al., 2009; Gomez et al., 2009; Sanz et al., 2005; Malbran et al., 2007; Bavbek et al., 2009; Harrer et al., 2010; Korosec et al., 2011; Steiner et al., 2011; Abuaf et al., 2012; Kim and Cho, 2012; Hagau et al., 2013; Phillips-Angles et al., 2016). Sensitivities varied from 0 (Harrer et al., 2010; Steiner et al., 2011) to 80% (Korosec et al., 2011) and specificities from 40 (Celik et al., 2009) to 100% (Gamboa et al., 2003a, 2004b; Hagau et al., 2013). Reasons may be manifold as single NSAIDs and different combinations of NSAIDs were tested and activation times ranged from 15 min to 3 h. In contrast to the negative BAT results, a most recent case report showed that basophils of an etoricoxib-hypersensitive patient were tested positive upon stimulation with the parent drug, although the authors excluded an involvement of drug specific IgE (Phillips-Angles et al., 2016). Here and in most other studies CD63 was the most commonly used activation marker. Introduction of CD203c by some groups (Bavbek et al., 2009; Celik et al., 2009; Abuaf et al., 2012) did not make results less ambiguous. Korosec et al. (2011) pointed out that a considerate pre-selection of most severe cases of DHRs may lead to an improvement in sensitivity and

specificity of BAT. Another important factor for investigating NSAID hypersensitivity may be the time interval between the incident and BAT which should be less than 18 months according to the EAACI position paper on BAT (Hoffmann et al., 2015) or less than 6 months according to Gomez et al. (2009). To put it all in a nutshell, due to the many contradictory data regarding sensitivity and specificity, potentially resulting from differences in gating protocols influencing basophil activation (Chirumbolo, 2014), further large-scale trials following harmonized BAT protocols including data interpretation are needed to determine suitability of BAT for diagnosis of NSAID hypersensitivity.

Basophil Activation in Hypersensitivity to Other Drugs and Some Remarks on Biologicals

Various other drugs causing immediate DHRs have been tested using BAT including atropine (Cabrera-Freitag et al., 2009), glatiramer acetate (Soriano Gomis et al., 2012), methylprednisolone (Aranda et al., 2010; Ben Said et al., 2010a), omeprazole (Gamboa et al., 2003b), the diuretic hydrochlorothiazide (Gamboa et al., 2005; Manso et al., 2010), or antihistamines (Caceres Calle and Fernandez-Benitez, 2004; Bobadilla-Gonzalez et al., 2011; Lee et al., 2011; Sanchez Morillas et al., 2011) to name a few. However, these reports, most of them case studies, are hard to judge from the perspective of validating BAT performance, as no experimental details were presented. Aranda et al. (2010) showed log-shifts in CD63 expression in the dot plots and indicated involvement of methylprednisolone-specific IgE as activation could be inhibited by wortmannin. Accordingly, Soriano Gomis et al. (2012) determined drug-specific IgE in their workup, however, no details on how BAT was conducted were given. Unequivocal BAT performance with 100% sensitivity and 87.5% specificity evidenced by log-shifts in CD63 expression shown in dot plots was reported for Gelofusine® hypersensitivity of 6 patients (Apostolou et al., 2006).

Adverse reactions to biologicals are increasing due to their expanding utilization, as reviewed recently (Corominas et al., 2014; Galvao and Castells, 2015). For instance, rituximab hypersensitivity with an incidence of 5–10% has been evaluated using BAT in a cohort of 18 B cell lymphoma patients (Piva et al., 2012). The authors reported successful discrimination between patients and controls based on CD63 expression at *in vivo* concentrations, however, no experimental details were presented. Very recently an interesting new approach to predict DHRs against cetuximab during cancer immunotherapy based on evaluating the decrease in cetuximab molecules on basophils after dissociation of IgE from FcεRI was reported (Iwamoto et al., 2016).

Notably, Werner J. Pichler has proposed a new classification for biologicals in five classes (α-ε), as biologicals differ in that they represent intact antigens themselves and are not metabolized like small molecular weight drugs (Pichler, 2006). We will thus not discuss ADRs against biologicals here.

CONCLUSIONS

Immediate DHRs present a complex phenomenon in regard to etiology. Similar clinical phenotypes are observed in "type I DHRs" with—according to the (pro-)hapten concept—drug-specific IgE against e.g., antibiotics, half of NMBAs, PP, or biologicals, and in "type I-like DHRs" elicited by alternative activation pathways as it is most likely the case with the other half of NMBAs, most RCM, platinum-containing chemotherapeutics, or NSAIDs. A close look on the CD63 upregulation pattern may help differentiating, as in type I DHRs the sudden basophil degranulation usually results in a log-shift of signal intensity. In contrast, no unequivocal conclusions on the underlying mechanism may be drawn when a "smear-like" CD63 upregulation is observed. In such cases, especially if basophil activation is weak, type I-like mechanisms independent of IgE cannot be excluded but bear the risk of false positive interpretation. We, therefore, recommend inclusion of plots/histograms in the publications to allow the reader evaluating the basophil activation profile. To circumvent bystander effects caused by interaction with other cells basophil purification protocols, as described recently (Steiner et al., 2016), may be considered, in particular when investigating more specific questions beyond diagnosis. Overall, the basophil response in BAT is influenced by many so far unpredictable factors which may impair the quality of results in certain cases (Chirumbolo, 2016).

Finally, we shall not forget that basophil activation test is an *in vitro* surrogate marker for a systemic reaction of the entire organism. This renders skin and/or provocation testing still of primary importance for diagnosis. Nevertheless, BAT has qualified a safe and suitable *in vitro* complement of *in vivo* testing in immediate DHRs, as most recently pointed out by a position paper of the European Network for Drug Allergy (ENDA) and the EAACI Drug Allergy Interest Group (Mayorga et al., 2016).

AUTHOR CONTRIBUTIONS

MS, AH, and MH were involved in the concept, literature screening, and writing of the article.

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