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Antibody–drug conjugates: prospects for the next generation

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The concept of a 'magic bullet' was first introduced by Paul Ehrlich in the early 1900s, he foresaw the advent of targeted therapies and the specific killing of harmful cells and/or microorganisms. However, these therapies were only used in the clinic after the second half of the 20th century with the development of specific monoclonal antibodies. To date, 13 antibody–drug conjugates (ADCs) are commercially available. Many advances have been made by modifying one or several of the three main components of an ADC, namely the antibody, the cleavable or non-cleavable linker or the payload, and by integrating conjugation chemistry. Despite these efforts, some problems have emerged and thus limit their effectiveness. New strategies could overcome these problems and identify the next generation of ADC.

Keywords: Antibody–drug conjugate; generation; drug:antibody ratio; payload diversification; aggregation; theranostics

Introduction

According to estimates by the WHO, cancer was the cause of nearly 10 million deaths worldwide in 2020, and represents the first or second most common cause of death before the age of 70 years in 112 out of 183 countries.[(p1),(p2)] During the 20th century, extensive research led to the discovery and development of cytotoxic compounds capable of rapidly killing cancer cells, which became central to standard-of-care treatments for various cancers. However, one of the notorious drawbacks of chemotherapy is the significant toxicity of these compounds which, in addition to damaging cancer cells, also damage healthy tissue. Chemotherapy can cause major side effects with serious consequences.[(p3)]

To overcome chemotherapy toxicity, several approaches have been assessed, including the identification of drug delivery systems with a high local cytotoxic efficacy but with a lower systemic toxicity.[(p4)] In the early 2000s, one of these novel approaches: antibody–drug conjugates (ADCs), consisted of joining one or more cytotoxic agent(s) onto a monoclonal antibody (mAb) via a linker, to specifically target the antigens on the surface of cancer cells. After decades of research, marketed ADCs have revolutionized the treatment of many types of cancers. For instance, brentuximab vedotin (Adcetris®), which is indicated for the treatment of CD30-positive Hodgkin's lymphoma (HL), was approved by the FDA in 2011 and then rapidly in 2012 by the European Commission (EC). Since the marketing authorization of these first ADCs, their development has been the subject of significant innovations for the production of the three components of an ADC: the antibody, the cleavable or non-cleavable linker and the bioactive payload, as well as for the discovery of new target antigens. More than 360 clinical trials (Phases 1 to 4) are currently being conducted on ADCs worldwide (studies under recruitment, active and not recruited study Phases: early Phase 1, 1, 2, 3 and 4), primarily to determine their safety and efficacy.[(p5)] According to a market research report by Strategic Market Research (SMR), the global market size for ADCs

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was US$3.51 billion in 2020 and is expected to reach US$13.15 billion by 2030, underlining ADCs as one of the fastest growing fields in oncology therapy.[p6]

Since the launch of the first ADC, Mylotarg®, in 2000, 13 ADCs have reached the market (Table 1). Blenrep® was withdrawn from the US and European markets in 2023. For the Committee for Human Medicinal Products (CHMP) of the EMA, the efficacy of Blenrep® was not confirmed by the results of the open-label Phase 3 DREAMM-3 study.[p7] Nevertheless, an interim analysis of the Phase 3 head-to-head DREAMM-8 trial (with progression-free survival as the primary endpoint), combining Blenrep® and pomalidomide plus dexamethasone, showed consistent efficacy in second-line and later treatment for relapsed or refractory multiple myeloma.[p8] Classifying ADCs into different generations is not an easy task considering their structural complexity. Some recent scientific reviews have nevertheless subdivided the currently marketed ADCs into three generations that differ according to their composition and the characteristics of each component. These subdivisions differ according to the scientific journal and the characteristics taken into account to determine these generations.[p9],[p10],[p11] The classification carried out by Fu *et al.* seems to be the most relevant because it considers each component with its different characteristics.[p11] Here, we present the main limitations of ADCs and the strategies currently being implemented for the development of the next generation of ADC.

Current limitations

Humanized antibodies and beyond

Humanized antibodies, generated from non-human species to closely match human antibodies, are the most used in currently marketed ADCs. Indeed, of the 13 ADCs on the market (Table 1), eight contain humanized antibodies, such as trastuzumab emtansine (Kadcyla®). Despite their non-human origin, these ADCs remain immunogenic and lead to the production of human anti-human antibodies (HAHA), albeit to a lesser extent than human anti-chimeric antibodies (HACA), arising from patients with autoimmune disorders.[p12] To limit immunogenic reactions, fully human mAbs have been developed and are used in the new generation of ADCs, including in enfortumab vedotin (Padcev®) and tisotumab vedotin (Tivdak®). More generally, for most oncology mAbs, it should be noted that there has been no identified clinically significant effect of binding or neutralizing anti-drug antibodies in terms of PK or safety.[p13] The typical size of non-human mAbs used in ADCs is ~150 kDa. This large size can limit the ability of the antibody and thus the ADC to penetrate target tissues and cells.[p14]

Heterogeneity of the drug load

The drug:antibody ratio (DAR) describes the average number of drug molecules conjugated to each antibody (Table 1). Interchain disulfide bridges, lysine and cysteine residues present on the surface of the antibody are the most commonly used binding modification sites on antibodies. The initial conjugation methods used led to fairly heterogeneous ADCs (first generation) and the use of more-specific conjugation methods has considerably reduced this heterogeneity (third generation). Of note, an unconjugated antibody fraction (DAR = 0) can remain following the conjugation step, and this can also limit the efficacy of the ADC, because it can compete with ADCs for the target antigen. Nevertheless, it should be noted that, in some cases, a carrier dose of unconjugated antibody can improve the PK properties of ADCs used in combination. Ponte *et al.* even demonstrated that an ADC with a lower DAR could behave like the above-mentioned combination.[p15]

The establishment of the DAR for each ADC is therefore essential and should reflect its homogeneity and heterogeneity.[p16] At present, however, documents provided by the FDA and the EMA only include the average DAR, which does not indicate the precise distribution of the loads for each ADC, which could affect the final therapeutic efficacy of the ADC. For example, unlike homogeneous ADCs (better antitumor activity, improved survival), conventional heterogeneous ADCs were not effective *in vivo* at treating glioblastoma multiforme (GBM).[p17] Thus, the homogeneity of the drug load of ADCs constitutes an important parameter for the development of future generations which will be more effective and more stable.

Given the complexity of ADCs in terms of structure and biological activity, the manufacturer will have to provide a large number of so-called 'critical quality attributes' (CQAs). Among the list of CQAs, in addition to the average DAR and drug load distribution, the glycoprofile, the amount of unconjugated antibody, the drug conjugation sites and the residual drug linker and related product proportions, in addition to high and low molecular weight species, and charge variants must be indicated.[p18] The preparation of a non-heterogeneous ADC in terms of drug load is an important objective from a regulatory point of view, in order to promote the marketing of a homogeneous final product.

DAR, hydrophobicity and clearance

The effectiveness of ADCs is also related to their hydrophobicity, itself related to the DAR. The potency of ADCs (i.e., their level of biological activity in a given system) increases *in vitro* with the DAR but decreases *in vivo* owing to faster plasma clearance of highly charged antibodies. Lyon *et al.* demonstrated this by studying the *in vivo* clearance of an anti-CD70 h1F6 antibody bound to highly charged monomethylauristatin F (MMAF) (DAR = 8).[p19] Compared with the unconjugated h1F6 antibody, the corresponding ADC was cleared faster. In parallel, at the same dose (2 mg/kg), the lesser loaded ADC (DAR = 4) showed greater activity than the highly loaded ADC (DAR = 8) with an almost three times greater decrease in tumor volume. A complementary work using polyethylene glycol (PEG) to reduce the hydrophobicity of homogeneous ADCs confirmed the relevance of this strategy to improve their stability.[p19]

Lipophilic payloads and aggregation

The conjugation of mostly lipophilic payloads can increase the hydrophobicity of mAbs, resulting in reduced stability as mentioned above and also in increased aggregation. Aoyama *et al.* carried out a study on the cytotoxicity of ADC aggregates in target-negative cells, by using Kadcyla® and Enhertu®.[p20] The results showed that aggregates formed under stressful conditions (agitation or heat stress) increased the cytotoxicity in HER2-negative cells, especially immune cells expressing the FcγR.

TABLE 1

Antibody–drug conjugates approved for market worldwide[a]

Trade name (company)	INN	Antibody			Linker		Payload (DAR)	Approved year	Indication
		Type (IgG subclass)	Target protein	Conjugation site	Type	Cleavable			
Mylotarg® (Pfizer)	Gemtuzumab ozogamicin	Humanized (IgG4)	CD33	Lys	Hydrazone	Hydrolytic cleavage	*N*-acetyl gamma calicheamicin (2–3)	2000 (FDA) withdrawn in 2010 reapproved in 2017[b] 2018 (EC)[b]	CD33 positive AML
Adcetris® [Takeda Pharmaceutical (EU)/Seagen (FDA)]	Brentuximab vedotin	Chimeric (IgG1)	CD30	Cys	mc-Val-Cit-PABC	Protease-cleavable	MMAE (4)	2011 (FDA) 2012 (EC)	Hodgkin's lymphoma
Kadcyla® (Roche)	Trastuzumab emtansine	Humanized (IgG1)	HER2	Lys	SMCC	Stable thioether linker	DM1 (3.5)	2013 (FDA & EC)	HER2-positive breast cancer
Besponsa® (Pfizer)	Inotuzumab ozogamicin	Humanized (IgG4)	CD22	Lys	Hydrazone	Acid-cleavable	*N*-acetyl-gamma-calicheamicin (6)	2017 (FDA & EC)	B-cell ALL
Polivy® (Roche)	Polatuzumab vedotin	Humanized (IgG1)	CD79b	Cys	mc-Val-Cit-PABC	Protease-cleavable	MMAE (3.5)	2019 (FDA) 2020 (EC)	DLBCL
Padcev® (Astellas Pharma)	Enfortumab vedotin	Human (IgG1)	Nectin-4	Cys	mc-Val-Cit-PABC	Protease-cleavable	MMAE (3.8)	2019 (FDA) 2022 (EC)	la/mUC
Enhertu® (Daiichi Sankyo)	Fam-trastuzumab deruxtecan	Humanized (IgG1)	HER2	Cys	mc-Gly-Gly-Phe-Gly	Lysosomal enzyme-cleavable	Deruxtecan (8)	2019 (FDA) 2021 (EC)	HER2-positive breast cancer
Trodelvy® (Gilead Sciences)	Sacituzumab govitecan	Humanized (IgG1)	Trop-2	Cys	CL2A	Acid-cleavable	SN-38 (7.6)	2020 (FDA) 2021 (EC)	la/mTNBC or mUC
Blenrep® (GlaxoSmithKline)	Belantamab mafodotin	Humanized (IgG1)	BCMA	Cys	Maleimido-caproyl	Non-cleavable	MMAF (4)	2020 (FDA & EC) withdrawn in 2023	RRMM
Akalux® (Rakuten Medical)	Cetuximab sarotalocan	Chimeric (IgG1)	EGFR	Lys	Not available	Not available	IRDye700DX (1.3–3.8)	2020 (PMDA)	Unresectable locally advanced or recurrent HNSCC
Zynlonta® (ADC Therapeutics)	Loncastuximab tesirine	Chimeric (IgG1)	CD19	Cys	mc-PEG8-Val-Ala-PABC	Protease-cleavable	SG3199 (2.3)	2021 (FDA) 2022 (EC)	(R/R) DLBCL
Tivdak® (Genmab/Seagen)	Tisotumab vedotin	Human (IgG1)	Tissue factor	Cys	Val-Cit	Protease-cleavable	MMAE (4)	2021 (FDA)	r/mCC
Aidixi® (RemeGen)	Disitamab vedotin	Humanized (IgG1)	HER2	Cys	mc-Val-Cit-PABC	Protease-cleavable	MMAE (4)	2021 (NMPA) 2021 (FDA)	LAGC or MGC
Elahere® (ImmunoGen)	Mirvetuximab soravtansine-gynx	Chimeric (IgG1)	FRα	Lys	Sulfo-SPDB	Protease-cleavable	DM4 (3.4)	2022 (FDA) 2024 (positive opinion adopted by the EMA's CHMP)	FRα-positive epithelial ovarian, fallopian tube or primary peritoneal cancer

Abbreviations: AML, acute myeloid leukemia; B-cell ALL, B cell acute lymphoblastic leukemia; BCMA, B cell maturation antigen; CHMP, Committee for Medicinal Products for Human Use; CL2A, PEG8- and triazole-containing PABC-peptide-mc (CAS No.: 2616704-22-2); DM1, maytansinoid derivative 1; DM4, maytansinoid derivative 4; DTPA, diethylenetriaminepentaacetic acid; EC, European Commission; EMA, European Medicines Agency; FDA, Food and Drug Administration; FRα, folate receptor-alpha; HER2, human epidermal growth factor receptor 2; HNSCC, head and neck squamous cell carcinoma; IgG1, immunoglobulin subclass 1; IgG4, immunoglobulin subclass 4; LAGC, locally advanced gastric cancer; la/mTNBC, locally advanced or metastatic triple-negative breast cancer; la/mUC, locally advanced or metastatic urothelial cancer; mc-Val-Cit-PABC, maleimidocaproyl-L-valine-L-citrulline-p-aminobenzoyloxycarbonyl; MGC, metastatic gastric cancer; MMAE, monomethyl auristatin E; MMAF, monomethyl auristatin F; NMPA, National Medical Products Administration; PMDA, Pharmaceuticals and Medical Devices Agency; r/mCC, recurrent or metastatic cervical cancer; (R/R) DLBCL, relapsed/refractory diffuse large B-cell lymphoma; RRMM, relapsed/refractory multiple myeloma; SMCC, *N*-succinylidene-4-(maleimethylene)cyclohexane-1-carboxylic acid; sulfo-SPDB, *N*-succinimidyl-4-(2-pyridyldithio)-2-sulfobutanoate; Trop-2, trophoblast cell surface antigen 2.

[a] All specific medical information for each approved drug can be consulted on the EMA (https://www.ema.europa.eu/en/medicines), FDA (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm), NMDA (https://english.nmpa.gov.cn/) and PMDA (https://www.pmda.go.jp/english/review-services/) websites.

[b] Reintroduction of gemtuzumab ozogamicin (GO) in the USA (2017) and its authorization in Europe (2018) were decided after a *meta*-analysis of five clinical trials. GO in combination with chemotherapy (e.g., daunorubicin) significantly improved overall survival at 5 years.

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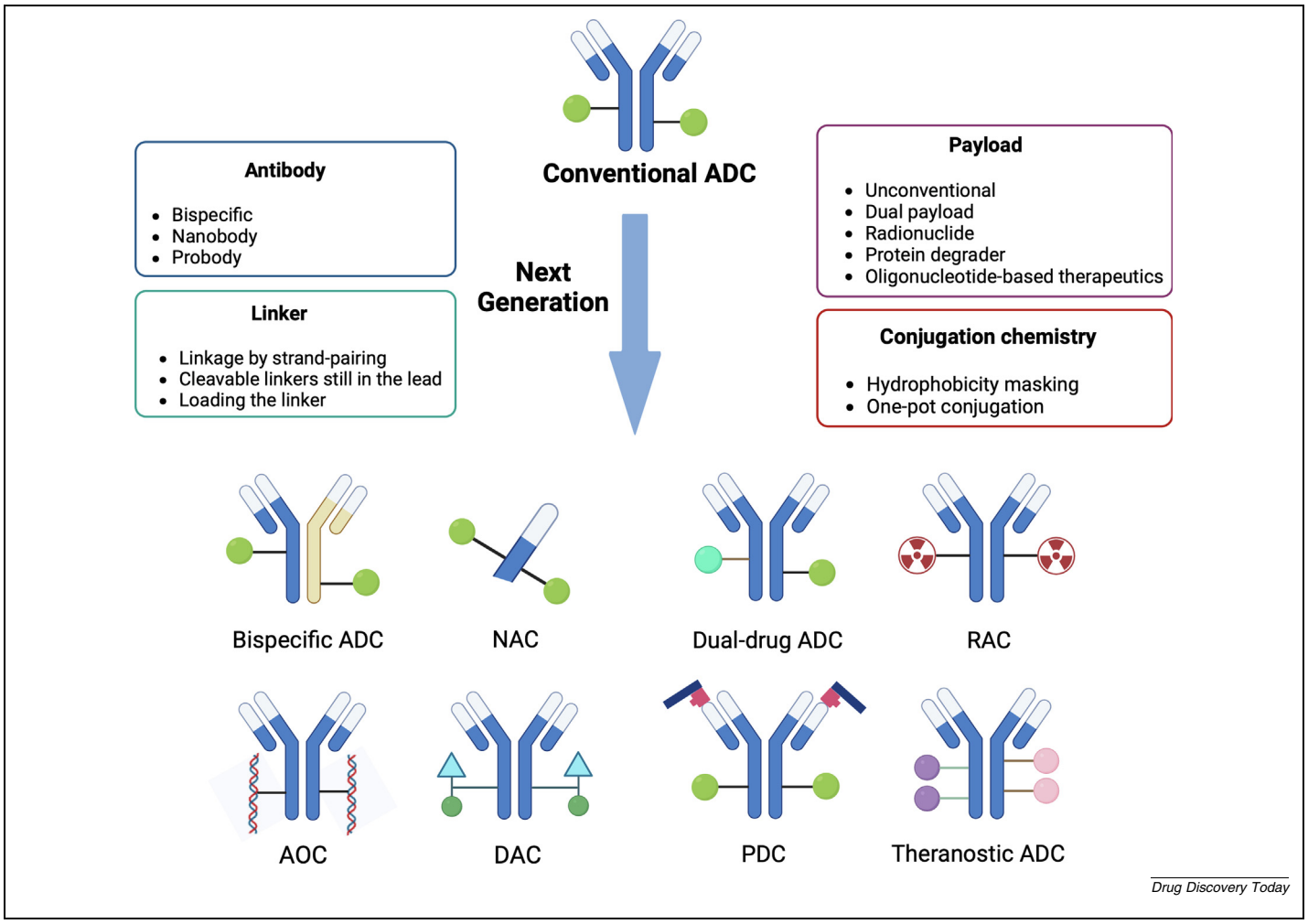

FIGURE 1

Exploring the next generation of antibody–drug conjugates (ADCs). A traditional ADC consists of three parts: antibody, linker and payload. Depending on the choice of antibody, linker, payload and conjugation chemistry, new ADCs are developed. New terms emerge such as bispecific ADC, nanobody–drug conjugate (NAC), dual-drug ADC, radionuclide–antibody conjugate (RAC), antibody–oligonucleotide conjugate (AOC), antibody-degrading conjugate (DAC), probody–drug conjugate (PDC) and theranostic ADC. Created with BioRender.com.

Resistance linked to tumor heterogeneity

The selection of the target antigen is a very important step to develop effective ADCs to act selectively on tumor cells. One of the mechanisms of resistance to cancer treatments is tumor heterogeneity highlighted by genomic analyses.[p21] A neoadjuvant Phase 2 clinical trial of T-DM1 (trastuzumab–emtansine) combined with pertuzumab was conducted to assess the impact of heterogeneity of HER2-positive tumors on the response to HER2-targeting therapies. The evaluation of patient biopsies included in the trial, as well as their results, showed that the main cause of treatment resistance was the fraction of ERBB2 nonamplified cells across the tumor. In fact, the pathological complete response (pCR) rate was 55% in the non-heterogeneous subgroup and 0% in the heterogeneous group.[p21] An additional study also based on T-DM1 revealed that consideration of increased baseline aneuploidy and potentially altered intracellular drug trafficking (e.g., solute transporters) is crucial to fully explain T-DM1 resistance.[p22] Despite the success experienced by ADCs, the limitations currently observed require continued efforts to improve the next generation of ADCs (Figure 1). There are multiple avenues for improvement, depending on the choice of the mAb, the payload and the linker, as well as the bioconjugation chemistry used for the final macromolecular assembly.

Antibody–antigen recognition and beyond

Bispecific antibodies

The use of bispecific antibodies is a promising strategy for the treatment of various cancers. For instance, blinatumomab (Blincyto®) is a bispecific CD19-directed CD3 T-cell engager that binds to CD19 (expressed on cells of B-lineage origin) and CD3 (expressed on T cells) used in the treatment of B-cell precursor acute lymphoblastic leukemia (ALL).[p23] Such a strategy could be applied to the development of new ADCs, linking a bispecific antibody that recognizes two different targets and a conventional payload. An illustration of this approach was made by de

Goeij *et al.* who tested the possibility of enhancing lysosomal delivery of ADCs by targeting a tumor-specific antigen in combination with an antigen that facilitates trafficking to the lysosomes.[p24] They designed a bispecific antibody (bsHER2xCD63his) composed of an arm targeting CD63, a ubiquitous protein present in intracellular compartments and on the surface of cells, and a binding arm HER2 which is a protein localized at the surface of cells and overexpressed in various cancers. They demonstrated that bsHER2xCD63his was more abundant in lysosomes compared with control antibodies that only target HER2 or CD63 after 16 h in SK-OV-3 cells. They then studied the cytotoxicity of a bispecific ADC composed of bsHER2xCD63-his conjugated to an antimitotic agent duostatin-3 with a cleavable valine–citrulline (VC) linker. A decrease in viable Colo 205 tumor cells (50 000 HER2/cell) treated with bsHER2xCD63his–ADC was observed compared with monospecific ADCs. These results indicate that the use of bispecific ADCs improves payload delivery and cytotoxicity.

Bispecific ADCs present several advantages such as improving selectivity toward target cells and promoting internalization by favoring a dual-antigen binding mode (e.g., with fast turnover receptors as prolactin receptor).[p25] However, the hurdles to overcome remain major. For example, in addition to developing site-specific conjugation methods and incorporating pharmacogenomics analysis in early clinical trials, other considerations for the design of bispecific ADCs must explore 'piggyback' and 'hijacking' transportation.[p25]

Nanobodies

Given the impaired penetration of ADCs of large size, new antibody formats have been developed to significantly reduce the size of the antigen-recognizing fraction.[p26] Indeed, nanobody–drug conjugates (NDCs) consist of a nanobody of smaller size than a mAb, albeit with similar properties regarding the payload. Based on this new approach, PEN-221 (≈2 kDa) was developed to treat small-cell lung cancer (SCLC) overexpressing somatostatin receptor 2 (SSTR2).[p27] PEN-221, a miniature drug conjugate, is composed of a SSTR2 agonist [Tyr3, Cys8]octreotate amide octreotate amide linked to the microtubule agent DM1 through a disulfide bond. Whalen *et al.* demonstrated that PEN-221 is effective in various *in vitro* and *in vivo* models with a dose-dependent response.[p27] Compared with octreotide and BT-984 (the non-binding SSTR2 scrambled control conjugate), it was demonstrated that PEN-221 was better internalized in the NCI-H524 model, which expresses high levels of SSTR2 at the cell membrane of tumor cells. In several mouse models of human SCLC xenografts expressing SSTR2,[p27] potent antitumor activity was observed. Conversely, no improved response occurred in the non-SSTR2-expressing model: Calu-6. Interestingly, low doses, about one-sixth (0.33 mg/kg) of the mouse MTD (2.0 mg/kg), caused significant tumor growth inhibition. These findings demonstrate the added value of miniaturized conjugates that penetrate solid tumor cells better, thus increasing the effectiveness of anticancer treatments. However, no miniaturized ADC has been approved in the past 10 years.

Beyond the targeted antigen

One of the main concerns in the development of cancer treatments is the heterogeneity that can occur within tumors. This heterogeneity is caused by stochastic genetic and epigenetic changes that confer hereditary phenotypic and functional differences to cancer cells. It was shown that the heterogeneous expression of the target antigen reduces the effectiveness of ADCs and is associated with poor survival in HER2-positive breast cancer.[p28] Some ADCs trigger antitumor activity against cancer cells located near the cells expressing the targeted antigen, which could address concerns linked to tumor cell heterogeneity. This property, called the bystander effect, is described in numerous reviews and is attracting increasing attention in the development of new ADCs.[p29] Many studies highlight the role of a cleavable linker and a hydrophobic payload for an effective bystander effect.

Suzuki *et al.* conducted a study to evaluate the intratumoral PK of [fam-]trastuzumab deruxtecan (T-DXd) marketed under the name Enhertu®.[p30] T-DXd is composed of a humanized anti-HER2 antibody and a highly membrane-permeable exatecan derivative (DXd) linked by a cleavable peptide-based linker. In 2019, T-DXd was first used in the treatment of HER2-positive unresectable or metastatic breast cancer. With phosphor-integrated dot (PID) imaging analysis, the researchers demonstrated the pharmacologic mechanism of T-DXd in human gastric cancer NCI-N87 cells and three human breast carcinoma cell lines (BT474, MCF7, MDA-MB-468). After 72 h, a strong distribution of DXd-PID in HER2-positive areas was observed which confirmed that the distribution of T-DXd in tumor tissue is dependent on the level of HER2 expression. Moreover, unlike trastuzumab distribution, T-DXd was more abundant in HER2-negative areas adjacent to HER2-positive areas. These results clearly illustrate the bystander effect of T-Dxd in the tumor microenvironment, encouraging further investigations into this phenomenon in the next generation of ADCs.

Probody and beyond

In the context of a protease-rich tumor environment,[p31] proteases can act as activators of prodrugs. This approach can also be applied to macromolecules such as antibodies. This involves preparing masked antibodies to minimize their interaction with healthy cells. Once the antibody reaches the protease-rich tumor environment, the deprotection step removes the protective mask and antibody–antigen recognition can take place.

Among published studies, Etxeberria *et al.*[p32] demonstrated the dual advantage of this strategy known as 'probody', namely effective antitumor efficacy combined with reduced toxicity of the anti-mouse CD137 agonist antibody 1D8 (1D8 probody therapeutic, Pb-Tx). In the clinic, urelumab, an anti-human CD137 agonist mAb, was related to severe liver-inflammation-related side effects. The construction of Pb-Tx with a protease-sensitive linker and a peptide mask has made it possible to significantly reduce liver toxicity. The work of Etxeberria *et al.*[p32] demonstrated that anti-CD137 Pb-Tx remains masked *in vitro* and *in vivo* in healthy tissues. Once the peptide mask had been

cleaved, the antibody was able to selectively recognize CD137 tumor antigens, ensuring the effectiveness of this prodrug system applied to macromolecules.

It is now possible to prepare a new type of ADC called a probody–drug conjugate (PDC). For example, Singh *et al.*[p33] developed CX-2029, a Pb-Tx conjugated to maleimido-caproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethyl auristatin E (MMAE). This PDC has the ability to deliver MMAE into the cells expressing CD71. They were thus able to demonstrate the *in vivo* efficacy of CX-2029 on a wide variety of PDX tumor models (e.g., pancreatic, diffuse large B-cell lymphoma (DLBCL), head and neck) and an acceptable *in vivo* toxicity profile compared to the corresponding anti-CD71 ADC. The latter ADC proved lethal in cynomolgus monkeys. This PDC is now being investigated in a Phase 1/2 clinical trial (NCT03543813) in patients with metastatic or locally advanced unresectable solid tumors or DLBCL.

Payload diversification

Unconventional payloads

For many years, microtubule-targeting and DNA-intercalating agents were at the forefront of ADC development. The recent approval and clinical success of trastuzumab deruxtecan (Enhertu®) and sacituzumab govitecan (Trodelvy®), two topoisomerase 1 inhibitor based ADCs, have highlighted the pertinence of conjugating unconventional payloads with different mechanisms of action. Among future developments in the ADC field, payload diversification is expected to play a key part as illustrated by a growing number of unconventional payload-conjugated ADCs currently at the preclinical and clinical stage. This recent review presents a comprehensive overview of validated, forgotten and newly developed payloads with different mechanisms of action (e.g., alpha amanitin).[p34] Controlled synthesis and full characterization of the first ADC containing ferroptosis inducer RSL3 fragment: trastuzumab, was completed, demonstrating a homogeneous DAR 8 conjugate.[p35] This ADC induced ferroptotic cell death through reactive oxygen species accumulation and increased the activity of doxorubicin.

ADCs are generally perceived to broaden the therapeutic index of their payloads. With the increasing use of highly cytotoxic payloads (e.g., amanitins), ADCs could prove to be an alternative. Preclinical trials are moving in this way. However, this therapeutic window expansion concept is called into question with results obtained during clinical evaluations. Colombo *et al.* clearly demonstrated that tolerated doses of ten ADCs do not differ significantly from those of related small molecules.[p36] They proposed various avenues of investigation to control the efficacy and the tolerability of ADC treatment, such as improving the drug-like properties of payloads (e.g., solubility, permeability, transporter substrate profile) and developing more predictive *in vitro* and *in vivo* models to enable better clinical translation.

Dual payloads

As for other anticancer treatments, resistance to ADCs by tumor cells is frequent. The mechanisms involved are multiple and induce the failure or reduction of the effectiveness of the treatment. For example, resistance to anti-HER2 ADCs can be caused by inter- and intra-tumor heterogeneity of breast tumors.[p37] To overcome this drug resistance, the use of an ADC with two payloads represents one of the most promising strategies in HER2-positive breast tumors with high heterogeneity and drug resistance. Yamazaki *et al.* developed dual-payload ADCs based on an anti-HER2 mAb with the N297A mutation.[p38] They used MTGase-mediated conjugation and orthogonal click reactions to conjugate the two payloads from the monomethyl auristatin family: MMAE and MMAF, to access three homogeneous dual-drug ADCs with defined DARs (2 + 2, 4 + 2 and 2 + 4). The *in vivo* antitumor activity of these new ADCs was evaluated in a xenograft breast tumor model characterized with HER2 heterogeneity. This new format was more effective than administering two single-payload ADCs. Compared with the ADCs conjugated to MMAE (DAR = 4 or 6), the ADC MMAE/F (DAR = 4 + 2) showed greater antitumor effects and greater tumor growth suppression. Thus, future generations of ADCs based on this technology can offer a therapeutic option to treat the many tumors that contain phenotypically and functionally heterogeneous cancer cells. By contrast, the industrial development of a dual-payload ADC will entail substantial additional financial costs, as well as strong regulatory pressure; module 3 of the common technical document will *de facto* have to contain several subdivisions of 3.2.S (payload 1, linker 1, payload 2, linker 2, payload-linker 1, etc.).

Radionuclides

The application of radioimmunotherapy in the development of innovative ADCs represents an interesting strategy in the treatment of solid and metastatic tumors. At the beginning of the 21st century (in 2002 an in 2003), two radionuclide–antibody conjugates (RACs): ^{131}I-tositumomab (Bexxar®) and ^{90}Y-ibritumomab tiuxetan (Zevalin®), were approved by the FDA for the treatment of non-Hodgkin's lymphoma (Table 2). In 2013, Bexxar® was withdrawn from the US market. Luo *et al.* also reported that ^{90}Y-ibritumomab tiuxetan in induction therapy of CD20-positive B cell non-Hodgkin's lymphomas increased overall response rate but did not improve progression-free survival, disease-free survival, overall survival and complete response rate compared with rituximab.[p39] Since 2024, Zevalin® has no longer been available in Europe. However, the design of new RACs is actively pursued. For example, in a Phase 1 study, Subbiah and his team evaluated dosimetry, safety and tolerability, PK and antitumor activity in patients with advanced solid tumors after treatment with a new type of ADC named ^{90}Y-FF-21101.[p40] It is composed of a chimeric immunoglobulin G (IgG1) mAb directed against P-cadherin and conjugated to a ^{90}Y radionuclide. Cadherins are transmembrane Ca^{2+}-dependent cell–cell adhesion molecules found in adherens junctions and contribute to the formation of solid tissues.[p41] The payload part composed of ^{90}Y emits radiation (beta particles) to directly attack cancer cells through DNA damage. The results of this study show a favorable safety profile with an estimate of the administered activity of ^{90}Y-FF-21101 below the applicable limits for external beam radiation therapy and great antitumor activity in several tumor types with an observed clinical benefit rate of 73%. These promising results confirm that radioimmunotherapy can be used in the development of new cancer treatments.

TABLE 2

Radionuclide-antibody conjugates (RACs) approved for market in Europe and the USA[a]

Trade name (company)	INN	Antibody			Linker		Payload (DAR)	Approved year	Indication
		Type (IgG subclass)	Target protein	Conjugation site	Type	Cleavable			
Zevalin® (Ceft Biopharma S.R.O.)	Ibritumomab tiuxetan	Murine (IgG1)	CD20	Lys	Thiourea-benzyl-DTPA	Non-cleavable	Yttrium-90 (^{90}Y) (not specified)	2002 (FDA) 2004 (EC) lapse of MA in 2024 in the EU	Non-Hodgkin's lymphoma
Bexxar® (GlaxoSmithKline)	Tositumomab and iodine ^{131}I tositumomab	Murine (IgG2a)	CD20	Aromatic ring	Tyrosine directly linked iodine-131 (^{131}I)	Non-cleavable	^{131}I (not specified)	2003 (FDA) withdrawn in 2013	Non-Hodgkin's lymphoma

Abbreviations: EC, European Commission; EU, European Union; DTPA, diethylenetriaminepentaacetic acid; MA, marketing authorization.

[a] All specific medical information for each approved drug can be consulted from the EMA (https://www.ema.europa.eu/en/medicines) and FDA (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm).

Protein degraders

With the aim of developing more-effective ADCs, new fields are being explored to select payloads providing a better therapeutic response. Proteolysis-targeting chimeras (PROTACs), first described by Sakamoto *et al.* in 2001,[p42] represent promising candidates in payload selection. They are composed of a ligand of a protein of interest (PoI) and a ligand of the E3 ligase linked by a spacer. Antibody-based PROTACs (AbTACs) are new entities that combine a mAb and a PROTAC connected by a chemical linker to maintain the stability of the molecule in the systemic circulation. After administration, the mAb recognizes the antigen on the tumor cell surface and the AbTAC undergoes internalization into the cell by receptor-mediated endocytosis (RME). Once inside the cell, the internalized AbTAC is then transported to the lysosome where the PROTAC payload is released by protease cleavage. The PROTAC then crosses the lysosomal membrane to reach the cytoplasm and binds, on the one hand, to PoI having a role in cellular signaling and, on the other hand, to the E3 ligase. This PoI then undergoes ubiquitination by the E3 ligase and degradation by the endogenous 26S proteasome. AbTACs and, more generally, antibody-degrading conjugate (DAC) systems, offer new therapeutic options and show significant *in vitro* and/or *in vivo* biological activities.[p43]

Pillow *et al.* described one of the first DACs composed of a mAb targeting C-type lectin-like molecule-1 (CLL1), which is overexpressed in acute myeloid leukemia (AML) patient myeloid blasts, and a bromodomain-containing protein 4 (BRD4) degrader (GNE-987).[p44] This compound exhibited a potent dose-dependent efficacy *in vivo* in the HL-60 and EOL-1 AML xenograft models following a single intravenous administration. Thus, improving DACs represents a relevant path for the development of future generations of ADCs.

Cotton *et al.* investigated the first AbTAC that degrades programmed death ligand 1 (PD-L1), a protein overexpressed in many cancers, which binds to the inhibitory receptor PD-1 on T cells and causes suppression of the T-cell response.[p45] They generated a bispecific IgG AbTAC that can simultaneously bind to RNF43 and PD-L1 (AC-1). AC-1 was studied on a triple-negative breast cancer cell line, MDA-MB-231, to observe whether AC-1 degraded PD-L1 on cells. After western blot analysis, they found that AC-1 can degrade PD-L1 at 10 nM after 24 h, whereas each component of the bispecific IgG had no individual effect on protein levels. They then tested AC-1 on other cell lines such as non-small-cell lung cancer (NSCLC) (HCC827) and advanced bladder cancer (T24). Likewise, treatment with AC-1 at 10 nM induced degradation of PD-L1 after 24 h. These data highlight the possibility of targeting cell surface proteins and a PoI using a fully recombinant bispecific IgG, via the AbTAC technology, with a biological construct that could mimic PROTACs.

Oligonucleotide-based therapeutics

It is also possible to use oligonucleotides, namely siRNA and antisense oligonucleotide (ASOs) as payloads. In recent years, there has been an acceleration in the approval of these RNA-based therapeutics.[p46] To prevent their massive uptake by hepatocytes, the use of mAb–oligonucleotide conjugates (AOCs) can prove to be an effective alternative. A recent study demonstrated that TfR1 mAbs (αTfR1) conjugated to siRNA and ASOs delivered the corresponding oligonucleotides to muscle cells.[p47]

Linker strategies

Linkage by strand pairing

In the field of AOCs, elegant work has been published on strand pairing between complementary oligonucleotides as a new linking approach.[p48] By example, Hsu *et al.* functionalized GC-rich ssDNA strands (18N) with succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC). The resulting 18N-MCC was then conjugated to the anti-HER2 IgG1 antibody (HTA101). Finally, HTA101-18N reacted with an 18NR–drug (e.g., hexachlorofluorescein, MMAE, DM1) to achieve strand pairing.[p48] Their methodology leaves open the possibility of using different linker formats for the 18NR–drug entity, such as 18NR-vc-MMAE and 18NR-MCC-DM1. Thus, they propose a modular ADC platform based on oligonucleotide strand pairing.

Cleavable linkers still in the lead

Of the 14 ADCs listed in Table 1, the great majority have a cleavable linker (11 out of 14). Structural analysis of each shows the success brought about by the introduction of an amino acid (AA) sequence included in a pluripartite heterobifunctional lin-

ker. There are thus eight ADCs with one or more AAs (e.g., lysine for Trodelvy®, valine–citrulline for Adcetris®, valine–alanine for Zynlonta® and glycine-glycine-phenylalanine-glycine for Enhertu®). Current developments, with the arrival of the latest ADCs (e.g., Tivdak®, Aidixi®), are moving in the same direction using a valine–citrulline sequence for example. To minimize the risk of premature release of payloads in blood circulation, new investigations are being carried out. Anami *et al.* have proposed adding a third AA: glutamic acid, to the traditional valine–citrulline sequence.[p49] The new sequence showed exceptionally high long-term stability in mouse and human plasma, as well as remarkable antitumor efficacy in xenograft models of mice with human breast cancer (JMIT-1 and KPL-4 cell lines). In addition, a new approach appears promising to provide spatial and temporal control over antibody activation. This is the use of exogenous triggers (e.g., light, ultrasound).[p50] Transposing this concept to ADCs will require the development of new dedicated projects.

The overall chemical nature of the linker is also essential to consider. Thus, the addition of a PEG spacer is an effective way of reducing the hydrophobicity of the ADC. Currently, Trodelvy® (SN-38, active metabolite of irinotecan) and Zynlonta® (SG3199, pyrrolobenzodiazepine dimer) use this option, with a PEG8-containing linker in both cases.

Loading the linker

To develop new homogeneous ADC with a high DAR of 18, Zacharias *et al.* described a THIOMAB XTEN–drug conjugate (TXC) composed of a mAb and a XTEN polypeptide chain linked via a cysteine residue.[p51] The payloads were linked by a VC dipeptide linker to the XTEN polypeptide. This approach was applied with different payload classes separately such as a microtubule-destabilizing agent (maytansinoid), a DNA monoalkylator (pyrrolobenzodiazepine monoamide) and an antibiotic [rifamycin analog dimethyl DNA31 (dmDNA31)]. After assessment of these TXCs, better antitumor and antibiotic efficacies were observed, unlike controls with a low DAR of 2. Thus, these results confirm that the use of TXCs improves antibody-mediated delivery (AMD) unlike conventional ADCs with a low DAR. By contrast, a Phase 1 dose-escalation study conducted with TXC DCDS0780A failed to validate this THIOMAB approach, because strong ocular toxicities were observed.[p52]

Conjugation chemistry

Hydrophobicity masking method

Recently, a hydrophobicity masking method was developed using a hydrophilic monodisperse polysarcosine (PSAR) drug-binding platform (PSARLink™).[p53] The addition of a variable chain of PSARn can enhance the therapeutic efficacy of high DAR ADCs. For example, in comparison with trastuzumab deruxtecan, hydrophobic interaction chromatography (HIC) profiles showed that ADCs based on this trastuzumab-exatecan-polysarcosine 10 (Tra-Exa-PSAR10) masking method eluted first and had a retention time comparable to native unconjugated trastuzumab. Additionally, an *in vivo* study demonstrated that Tra-Exa-PSAR10 has the same PK profile as unconjugated native trastuzumab. Indeed, the most potent antitumor activity was observed with Tra-Exa-PSAR10 compared to trastuzumab deruxtecan.[p54] These results therefore highlight the importance of hydrophilic components in the structure of a conventional ADC that allow a favorable PK profile and better *in vivo* activity. A FRα-targeting ADC based on the PSARLink™ platform, linked to a potent topoisomerase I inhibitor via a dipeptide cleavable linker (MBK-103/LY4170156), provided convincing results in preclinical evaluations and a Phase 1 clinical trial testing LY4170156 began in May 2024.[p55]

One-pot conjugation

Site-specific conjugation is a major issue in the field of ADCs. Among all those developed, one stands out. This is the AJICAP™ technology.[p56] This methodology was successfully used to modify Lys248 of native antibodies (e.g., IgG1, IgG4) resulting in homogeneous ADCs (DAR = 2). Prior to the final step (payload conjugation), this methodology encompassed three stages: peptide conjugation, linker cleavage and reoxidation. The linker cleavage (reduction step) between the antibody and the AJICAP peptide reagent involved the use of tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) to introduce SH groups on Lys248. However, this step could also cause the cleavage of the disulfide bonds of interchain cysteines in antibodies, which then required a third stage of reoxidation. Moreover, application of this methodology led to the formation of aggregates (5–10%), limiting wider application.[p57]

Fujii *et al.* have therefore developed a new one-pot approach, called AJICAP second-generation.[p58] This involved the evaluation of different AJICAP reagents. A thioester-based strategy identified two different AJICAP peptide reagents. The overall result of the study confirmed the preparation of conjugates based on Lys248 but also Lys288. The 20 ADCs prepared (varying in mAb, linker and payload) have remarkable homogeneity (1.8 < DAR by HIC < 1.9) and a very low aggregation rate (between 1.8 and 3.8%). This novel methodology is also compatible with antibody fragments and polyclonal antibodies. Access to new, non-traditional ADCs, as mentioned above (e.g., miniaturized ADCs, AOCs, DACs), will enable us to expand the scope of clinical investigations more rapidly, to treat cancers but also other pathologies.

Theranostics

More-efficient ADCs might rely on the integration of a theranostic payload to improve disease monitoring and treatment. Su *et al.* developed ADC H-233, composed of a mAb (trastuzumab) linked to the L-233 theranostic payload by an acid-cleavage carbonate linker.[p59] The payload includes a coumarin probe 7-nitro-3-hydroxyethyl-coumarin (7-NHC) and MMAE. Experiments carried out on the SKOV3 cell line demonstrated the efficacy of ADC H-233 in two stages.[p59] After internalization of H-233 into cells, L-233 is released via acid lysosomal conditions. Owing to the high expression of nitroreductase in the tumor microenvironment, the nitro group of the 7-NHC probe is then reduced to 7-amino-3-hydroxy-coumarin (7-AHC), resulting in rapid electron transfer and release of 7-AHC (fluorescence turn-on) and MMAE (tubulin polymerization inhibitor). The results of the *in vitro* study showed that L-233 can provide a direct and real-time monitoring method for ADC payload release. However, L-233 needs to be optimized to develop a theranostic payload with a detectable long wavelength *in vivo*. Knutson *et al.* developed a dual-labeled fluores-

cent ADC (α-CEA-680-PTX) composed of a mAb specific for a carcinoembryonic antigen (CEA) biomarker conjugated to paclitaxel and near-infrared (NIR)-modified PEGylated fluorophore (DyLight™ 680-4xPEG).[p60] They initially performed *in vitro* tests to assess the impact of fluorescent labeling on ADC function. On CEA-positive (BxPC-3) cell lines, α-CEA-680-PTX showed almost identical intensity to the unconjugated fluorescent antibody to paclitaxel (α-CEA-680) and better kinetics of internalization with a constant number of spots detected after 8–72 h compared with α-CEA-680. Then, they assessed the *in vitro* cytotoxicity of the dual-labeled fluorescent ADC using the MTT assay protocol for cell viability. This ADC was more potent than the unconjugated antibody (α-CEA-PTX) and this difference is probably caused by the increase in the DAR of the α-CEA 680-PTX, which is 20% higher than that of α-CEA-PTX. Finally, they conducted an *in vivo* evaluation on a mouse tumor xenograft model to determine the cytotoxicity of the fluorescent ADC *in vivo*. Compared with the control (PBS) and free paclitaxel, α-CEA-680-PTX had a better efficacy at decreasing tumor volume. Thus, these theranostic payloads could improve real-time disease monitoring during treatment and improve patient care.

Concluding remarks

The DAR remains an essential element in the field of ADCs, yet official documents from the competent authorities, such as the FDA and the EMA, only indicate an average DAR, which only partially reflects the homogeneity and heterogeneity of the ADCs. Thus, in addition to the average DAR, it would be relevant to systematically include the distribution of the drug load for each ADC. As mentioned above, homogeneity of ADCs affects their therapeutic efficacy.[p17] Unfortunately, homogeneity is not an absolute guarantee of success.[p61]

Currently, all the commercially available ADCs have been approved for anticancer indications such as urothelial cancer, certain leukemias and breast cancer. According to the WHO, >2.2 million cases of breast cancer were recorded in 2020. Recently, a study focused on the expression status of the HER2 protein on the surface of cancer cells by immunohistochemistry (IHC) and showed that it was highly heterogeneous within the same sample. Hence, defining the HER2 status of a patient is an important step in determining treatment.[p62] In addition, Wu *et al.* used an AI algorithm to increase the accuracy and consistency of interpreting HER2 IHC results (e.g., 0 and 1+ assessments).[p63] Among the results obtained, it can be noted that the AI-based technology identified patients with HER2-low breast cancers who might respond better to treatment with the ADC trastuzumab deruxtecan (Enhertu®).

Moreover, the therapeutic approach using ADCs is also useful for the development of therapies for non-oncological diseases. Several new ADCs are currently undergoing preclinical and clinical studies.[p64] For example, DSTA4637S, a THIOMAB ADC, is composed of a human anti-β-wall teichoic acid (β-WTA) mAb linked to a novel antibiotic 4-dimethylaminopiperidino-hydroxybenzoxazino rifamycin (dmDNA31) via a protease-cleavable VC linker.[p65] The authors showed that after DSTA4637S bound to β-WTA, a major component of the cell wall of *Staphylococcus aureus*, the corresponding complex was phagocytosed and after specific cleavage the antibiotic dmDNA31 killed *S. aureus*. DSTA4637S could therefore overcome the emergence of methicillin-resistant *S. aureus* (MRSA) strains because it has a different mechanism of action to conventional antibiotics. After promising results with regards to safety, tolerability and PK during the Phase I clinical study,[p66] clinical investigation on DSTA4637S is ongoing in patients with *S. aureus* bacteremia.[p67] No Phase 2 trial was initiated with DSTA4637S. Other therapeutic areas are currently being explored with ADCs, including immune-mediated inflammatory diseases[p68] and Alzheimer's disease.[p69] To date, the only approved ADCs are used in oncology.

In the coming months, three ADCs should receive marketing authorization to treat various forms of NSCLC in adults, namely datopotamab deruxtecan (humanized anti-TROP2 IgG1, tetrapeptide-based cleavable linker, exatecan derivative, DAR = 4), patritumab deruxtecan (fully human anti-HER3 IgG1, tetrapeptide-based cleavable linker, exatecan derivative, DAR = 8) and telisotuzumab vedotin (humanized bivalent anti-c-Met IgG1, cleavable dipeptide linker, MMAE, DAR = 3.1).[p70] The scope of investigations around ADCs is vast, offering incredible prospects in terms of the precision, efficacy and tolerability of future treatments. [p71],[p72]

CRediT authorship contribution statement

Meriem Grairi: Writing – review & editing, Writing – original draft, Resources. **Marc Le Borgne:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization.

Data availability

No data was used for the research described in the article.

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Conflicts of interest

No interests are declared.

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