The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy

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The aim of this review is to integrate non-exhaustive relevant data on the phenotype of human plasma cells (PC), including normal, reactive and malignant (multiple myeloma, MM) PC. This review focuses on (i) a universal marker of both normal and malignant plasma cells, CD138; (ii) markers related to malignancy i.e., CD19, CD27, CD28, and CD56; (iii) markers associated with signaling and severity of MM (CD45, CD221). Finally, this review presents data from normal PC up to human myeloma cell lines in order to: (i) define different entities of MM based on expression of CD19, CD20, CD27 and CD117; and (ii) identify new therapeutic targets.

Key words: plasma cells, phenotype, myeloma cells.

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Phenotype establishment is used for the diagnosis of several hematopoietic malignancies such as leukemia, but not for multiple myeloma (MM). However, phenotyping is a very sensitive and powerful approach, useful for both diagnosis and clinical follow-up. Establishing the phenotype of myeloma cells and its difference from that of normal PC has proven highly useful for (i): clearly identifying and characterizing malignant cells;[1-3] (ii) identifying prognostic markers;[4-7] (iii) preventing a false diagnosis of MM ;[8] (iv) evaluating minimal residual disease;[9] (v) suggesting antibody-based targeted therapies;[6,10-17] (vi) defining targeted downstream signaling therapies;[18-19] and (vii) computerizing the myeloma cell growth and differentiation through CD45 intraclonal hierarchy.[20] This review presents data on the phenotype from normal and reactive PC, as well as from primary and immortalized myeloma cells, in order to summarize how the study of the myeloma cell phenotype has brought up new concepts by identifying different patient entities as well as by establishing an intraclonal hierarchy. This approach lays the bases for new phenotype-dependent treatments for patients with MM.

Phenotype of normal and malignant PC: universal PC marker, markers associated with malignancy, markers of severity

A universal marker of normal and malignant plasma cells: CD138 (syndecan-1)

The lack of specific molecules for PC and MM hampered the establishment of MM phenotypes and sometimes even the recognition of tumor cells. In the 1980s, J Wijdenes obtained a myeloma-specific monoclonal antibody, B-B$_4$, by immunizing mice with the U266 human myeloma cell line (HMCL). This monoclonal antibody was first used for the purification of myeloma cells from the bone marrow of patients with MM.[21-22] Then, by flow cytometry, we demonstrated that B-B$_4$ specifically recognized both malignant and normal PC,[1] these latter being known to express brightly CD38.[23] B-B$_4$ monoclonal antibody enabled the establishment of MM phenotypes through the identification of myeloma subpopulations. In 1996, J. Wijdenes demonstrated that B-B$_4$ recognized syndecan-1 or CD138, a molecule specific to mouse PC.[24-25] CD138 is a molecule belonging to the heparan sulfate family. In human hematopoietic cells, CD138 expression is restricted to both PC and myeloma cells. In the mouse, syndecan-1 is also expressed by B-cell precursors.[25] In the human, apart from cells of the hematopoietic system, epithelial cells, mesenchymal cells and carcinomas express CD138. Syndecan is a Greek word meaning *stick together*. Syndecan-1 has a long extracellular domain that binds to soluble molecules (growth factors e.g. EGF, FGF, HGF) and to insoluble molecules (e.g. collagen, fibronectin) through heparan sulfate chains.[26-27] CD138 also mediates cell-cell adhesion through heparin-binding molecules expressed by adjacent cells. It has been recently shown that CD138 has a role as a co-receptor for numerous growth factors of myeloma cells (Baff/April, EGF).[28] Studies of PC differentiation show that CD138 must also be considered as a differentiation antigen.

Indeed, CD138 expression appears after the plasmablastic stage. CD138- plasmablasts are PC progenitors that differentiate into CD138^{+} PC precursors retaining some proliferative ability before final maturation into non-dividing CD138^{++} PC.[29-30] Although plasmablasts are rare and transient *in vivo*, they become easily detectable in the context of reactive plasmacytoses, which are transient peripheral expansions of both PC progenitors and precursors.[29,31]

Syndecan-1 also exists as a soluble protein resulting from membrane shedding. It is well known that apoptotic normal and malignant PC lose CD138 expression.[32] In patients with MM, soluble syndecan-1 is found in plasma and is a prognostic factor.[33-34] Soluble CD138 is believed to reflect both the tumor mass but also the apoptosis index (spontaneous or drug-induced).

All normal and malignant PC express CD38 and CD138 (Table 1). However, as illustrated in Figure 1 the level of expression is different and allows normal PC to be distinguished from malignant PC: myeloma cells express more CD138 but less CD38 than do normal PC (*unpublished data*). We have never observed viable CD138^{-} myeloma cells whereas CD38^{-} myeloma cells exist (two cases out of around 1,000 phenotypes of patients at diagnosis or relapse, *personal data*). Moreover, all HMCL express CD138 (29 out of 29) but not all express CD38 (4 out of 29 are CD38^{-}) (Table 3).

The immortalization of primary myeloma cells into HMCL remains a rare event. In almost all cases, HMCL are derived from patients with terminal disease involving extramedullary invasions of peripheral blood, pleura or peritoneum.[35-36] Some of the cell lines derived from patients with MM are not HMCL but B-cell lines transformed by Epstein-Barr virus (EBV) (e.g. IM9, ARH-77, MC/CAR). HMCL and B-EBV cell lines have globally opposite phenotypes (for example B-EBV cell lines lack both CD138 and CD38), as previously described.[36-38]

Table 1. A comparison of the phenotypes of normal, reactive and malignant PC.

	Normal (BM)	*Reactive (PB)*	*MM (BM)*	*PCL (PB)*	*HMCL*
CD138	+++	+++ and –	+++	+++	+++
CD38	+++	+++	+++	+++	+++
CD28	–	–	+	+	+
CD56	–	–	+++	–	+ or –
CD19	+	+	–	–	–
CD27	+	+	+ or -	–	–
CD45	+	+	–	–	+ or –

This table illustrates the most frequent phenotype of PC (– means not expressed: + expressed, +++ strongly expressed). The table is a synthesis of previously published data.[1-2,23-24,29,35,39,44,46,51,59] MM: multiple myeloma; PCL: primary plasma cell leukemia; BM: bone marrow; PB: peripheral blood; HMCL: human myeloma cell line.

Table 2. Frequencies of CD56, CD28, CD19, CD27 and CD20 expression.

	CD56	*CD28*	*CD19*	*CD27*	*CD20*
Number of patients analyzed	368	335	362	146	209
Number of positive	287	160	9	74	29
Percentage positive	78.0	47.8	2.5	50.7	13.9

Expression of CD56, CD28, CD27 and CD20 was evaluated in 368 MM patients at diagnosis. Patients were considered positive when at least 33% of myeloma cells were positive. Phenotype was evaluated in a three-color (CD38, CD138) or four-color (CD38, CD138, CD45) assay. This table is an update of previously published data.[1-2,7,13]

Markers associated with malignancy: aberrant expression of CD56 and CD28 but lack of CD19 and CD27

Aberrant expression of CD56 and/or CD28 is almost always observed in MM (94% of MM, n=336). As compared to normal PC, myeloma cells overexpress CD56 (78%, n=368), a marker of NK cells[1-2,39] (Figure 1 and Table 2). However, myeloma cells circulating into the peripheral blood usually lack CD56, whereas myeloma cells located in pleural or ascitic effusions express CD56.[1,2,4] It has not been elucidated whether CD56 expression in relation to location is the consequence of down- and up-regulation of CD56 expression or the result of preferential location of subpopulations in relation to CD56 expression. The lack of CD56 expression is currently associated with a lack of osteolysis. Indeed, in spite of a higher bone marrow myeloma infiltration, patients with CD56^{-} MM have fewer, if any, osteolytic lesions.[4,40-41] Lack of CD56 is associated with a poorer outcome.[42,43] CD56 is an adhesion molecule mediating homotypic interactions between myeloma cells themselves or with osteoblastic cells. CD56^{-} myeloma cells seem to be less toxic for osteoblasts (prevention of clump growth related to lack of CD56). On the other hand, lack of CD56 expression is associated with λ isotype.[39] CD56 is very frequently overexpressed in bone marrow (78% of patients at diagnosis), but far less so in extramedullary blood sites (8 out of 31, 26%).[4] Similarly, CD56 is only overexpressed in 8 of 29 HMCL (27%). Indeed, the HMCL phenotype is not similar to that of myeloma cells from patients at diagnosis, but rather resembles the phenotype of myeloma cells from patients with terminal extramedullary disease.[35,36]

CD28, a T-cell specific marker, is aberrantly expressed by primary myeloma cells i.e., 47.8%, n=335.[1,44] CD28 is not expressed by normal plasma cells.[1] Myeloma cells express one co-receptor of CD28, CD86, but not the other one, CD80.[44] CD28 is not involved in myeloma proliferation and survival (*personal data*), but CD28 triggering induces chemokine secretion.[45] Expression of CD28 is associated with reduced event-free survival but not overall survival.[43] Expression of CD28 increases with disease progression since its expression frequency increases with relapse.[44] Furthermore, CD28 is expressed by all HMCL,

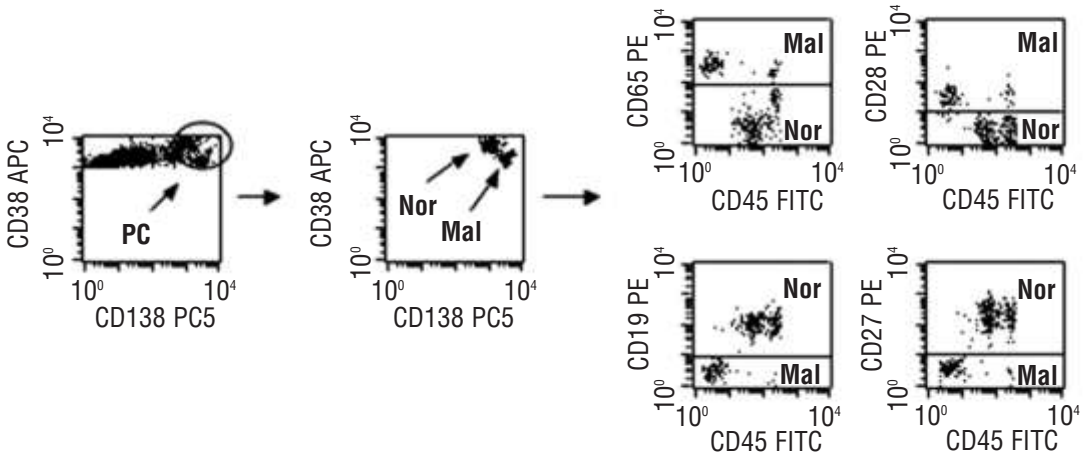

Figure 1. Figure 1 represents the phenotypes of normal and malignant plasma cells of a MM patient after treatment. Bone marrow mononuclear cells were stained in a four-color assay with CD38, CD138, CD45 and CD19 or CD56 or CD28 or CD27 monoclonal antibodies as previously described.[13] PC were identified by co-expression of CD38 and CD138 (which is the best combination for PC identification) and their phenotypes established on gated cells (first cytogram). Malignant PC (Mal) express a lower level of CD38 but higher level of CD138 than do normal PC (Nor). Their respective phenotypes are opposite: malignant PC are $CD19^-CD27^-CD28^+CD56^+$ and normal PC are $CD19^+CD27^+CD28^-CD56^-$. In this sample, normal and malignant PC represented 0.1% and 0.05% of mononuclear cells, respectively.

Table 3. Human myeloma cell lines.

	CD138	*CD38*	*CD56*	*CD28*	*CD19*	*CD27*	*CD45*	*CD20*	*Isotype*	*Sample*
ANBL-6	+	+	–	+ (4)	–	–	–	–	l	PB
BCN	+	+	–	+ (45)	–	–	–	–	Gk	PB
JIM3	+	+	–	+ (10)	–	–	–	–	A	PE
JJN3	+	+	15% (100)	15% (10)	–	–	65% (4)	–	Ak	PE
KMS11	+	+	+ (1.3)	+ (31)	–	–	–	–	Gk	PE
KMS12BM	+	+	+ (1.5)	+ (1.5)	–	+ (20)	–	–	NS	BM
KMS12PE	+	+	–	+ (18)	–	–	–	–	NS	PE
KMS18	+	+	–	+ (97)	–	–	–	–	Al	PB
K620	+	–	5% (23)	+ (3)	40% (13)	–	–	50% (17)	Gl	PB
LP1	+	+	36% (5)	61% (10)	–	–	–	–	Gl	PB
L363	+	+	43% (11)	+ (19)	–	–	–	–	NS	PE
MDN	+	+	+ (121)	+ (26)	–	–	+ (6)	–	Gk	PB
MM1S	+	+	50% (10)	+ (20)	–	–	–	–	Al	PB
NAN1	+	+	91% (12)	+ (10)	–	–	–	16% (12)	Ak	PE
NAN2	+	+	+ (1.5)	+ (24)	–	–	87% (5)	–	Gl	PB
NAN3	+	+	15% (5)	+ (14)	–	–	–	–	Ak	PE
NAN4	+	+	+ (1.6)	+ (4)	–	–	+ (5)	–	Ak	PB
NAN5	+	+	+ (57)	+ (4)	–	–	+ (49)	–	Gk	AF
NAN6	+	–	–	+ (52)	–	–	70% (18)	–	Ak	PE
NCIH929	+	+	+ (64)	+ (42)	–	–	18% (4)	–	Ak	PE
OPM2	+	+	+ (125)	+ (39)	–	–	–	–	Gl	PB
RPMI8226	+	+	+ (1.3)	+ (116)	–	–	–	–	Gl	PB
SBN	+	+	+ (4)	+ (9)	–	–	–	–	Al	PE
U266	+	–	–	+ (134)	–	–	89% (5)	–	El	PB
XG1	+	+	24% (10)	+ (27)	–	–	+ (20)	–	Ak	PB
XG2	+	+	+ (1.3)	+ (30)	–	+ (3.5)	+ (5)	–	Gl	PE
XG5	+	+	–	+ (24)	–	+ (1.5)	-	–	l	PB
XG6	+	–	–	+ (49)	–	–	+ (109)	–	Gl	PB
XG7	+	+	+ (6)	+ (41)	–	–	+ (2)	–	Ak	PB

Expression of each molecule is characterized by the percentage of the positive population (– means not expressed, + expressed by 100%, otherwise the % of positive cells is given) and the level of intensity (expressed as the ratio of fluorescence divided by the fluorescence of an isotypic control). Positive expression was considered to be present when the ratio was >1.2. Phenotype was evaluated in a single color assay. Part of these data have been previously published.[19,35-37] BCN, MDN, SBN, NAN1-6 were derived in our laboratory. HMCL ANBL-6, BCN, MDN, SBN, NAN1-6 and XG1-7 are cultured with IL-6. AF ascitic fluid, BM bone marrow, PB peripheral blood, PE pleural effusion. Isotype: l: lambda; k: kappa; A: IgA; G: IgG; E: IgE, NS: non secreting.

thereby being a universal HMCL marker, like CD138. A lack of CD19 and CD27 is frequent in MM. Lack of CD19 is observed in almost all patients (97.5%, n=362) and constitutes a marker of PC malignancy. Normal PC retain some CD19 expression (although weaker than that of B cells) but a subpopulation may lack CD19 expression. The role of the absence of CD19 expression, if any, has not yet been elucidated. As in primary myeloma cells, CD19 is very rarely expressed in HMCL (1 out of 29, i.e. K620).

In the B-cell lineage, CD27 is a memory marker since its expression is restricted to germinal center cells, memory cells and plasma cells. Gene expression profile studies of normal PC and myeloma cells have identified CD27 as being one of the most significant genes lost by myeloma cells.[46] However, half of MM retain CD27 (51%, n=146) and its expression is associated with a better prognosis.[7,47] CD27 expression is lost with myeloma progression. CD27 and its ligand, CD70, belong to the TNFR/CD40/TRAILR family. As for the other family members, triggering CD27 may induce either apoptosis or survival. In MM, CD27 triggering has been reported to induce either drug resistance in primary plasma cell leukemia or discrete apoptosis in CD27-transfected HMCL.[48-49] *In vivo*, CD70 is expressed by activated B cells and T cells. Therefore, lack of CD27 expression in myeloma cells could favor an escape from the immune system. Most HMCL lack CD27 expression (90%, n=29) although three of them express CD27 either weakly (XG2 and XG5) or even strongly (KMS12BM). As shown in Table 1, the global phenotype of PC allows identification of each type of PC i.e., normal, reactive and malignant, in relation to their location (peripheral blood versus bone marrow).

The phenotype helps to distinguish reactive plasmacytosis (polyclonal $CD19^{+}CD27^{+}CD28^{-}CD45^{+}$) from plasma cell leukemia (monoclonal $CD19^{-}CD27^{-}CD28^{+}CD45^{-}$) especially when reactive plasmacytoses are massive and associated with other failures (low platelet counts, low hemoglobin level) reminiscent of the presentation of plasma cell leukemia.[8,31]

Markers of disease severity: lack of CD45 and overexpression of CD221 (IGF1R). CD45 hierarchy and proliferation

Lack of CD27 or CD45 or overexpression of CD221 has been shown to be associated with a more adverse prognosis.[5-7] CD45 is a tyrosine phosphatase broadly expressed by hematopoietic cells. During PC differentiation, CD45 expression decreases. In non-malignant PC, CD45 is brightly expressed by all tonsillar, circulating or reactive PC.[50,51] Labeling index (LI) analysis shows that all these PC are proliferating (LI=15% and >30% for reactive PC). In bone marrow, CD45 is expressed by a subpopulation of PC and the proliferation (measured by LI) is restricted to this $CD45^{bright}$ compartment.[51] The association between CD45 and LI is confirmed by Bcl-2, since Bcl-2 is inversely correlated to proliferation (evaluated by LI or Ki67 staining) and also to CD45 expression.[51,52] Therefore, CD45 down-regulation is associated with increased maturation and proliferation arrest (confirmed by *in vitro* PC generation, *personal data*). In contrast to normal PC, myeloma cells show mainly CD45 weak or negative expression. However, like normal bone marrow PC, myeloma cells are a mix of a small $CD45^{bright}$ compartment and large $CD45^{low/neg}$ compartment.[20,50,51,53,54] Based on LI analysis, we and others identified this $CD45^{bright}$ minor compartment as being the proliferating myeloma compartment.[51,53] Bcl-2 and LI are also inversely correlated in MM cells.[52,55] We and others further demonstrated that the minor $CD45^{bright}$ compartment co expresses LFA-1.[51,54]

Of note, this compartment is directly dependent on interleukin-6 (IL-6) to grow through CD45 regulation. Indeed, Kulas and Kawano identified CD45 as a phosphatase required for IL-6-mediated growth.[20,56-58] CD45 dephosphorylates an inhibitory tyrosine on lyn that allows for its own activation and for IL-6-induced cell growth. Although both compartments are always present *in vivo*, HMCL are primarily either $CD45^{-}$ (n=17, 60%) or $CD45^{+}$ (n=7, 25%) and only few HMCL have both $CD45^{-}$ and $CD45^{+}$ populations (n=5, 15%).

The tyrosine phosphatase/kinase ratio: CD45 and CD221. Targeting IGF-1/AKT signaling

From a clinical point of view, CD45 expression on the major compartment (low versus negative) is a powerful prognostic factor. Indeed, we showed that $CD45^{-}$ patients have a very poor survival prognosis when compared to $CD45^{+}$ patients.[5] Overall, MM proliferation in $CD45^{-}$ patients is double that in $CD45^{+}$ MM patients (*unpublished data*). CD45 impairs the activation of the AKT pathway by insulin-growth factor-1 (IGF-1) or insulin. The lack of CD45 expression then allows a response to IGF-1, i.e. growth through the AKT pathway.[19,56] IGF-1R (CD221) is aberrantly expressed in myeloma cells when compared to normal and reactive PC. Moreover, patients with a high level of IGF-1R expression have a shorter survival.[6]

Over-expression of IGF-1R is associated with t(4;14) and lack of CD45 expression. Due to the lack of CD45 combined with a high level of IGF-1R expression, IGF-1 signaling should be favored in these patients. Moreover, recent data show that the decrease of CD45 expression in $CD45^{+}$ HMCL by shRNA enhances IGF-1 activation of AKT (*Descamps et al., in press*). Thus, because CD45 is a negative modulator of IGF-1 signaling but a positive one of IL-6 signaling, this tyrosine phosphatase is an essential regulator of MM cell growth and its expression (or its lack of expression) by myeloma cells is a critical determinant of the biology of MM.

Markers defining MM subsets: CD19, CD20, CD117. Correlation with PC morphology, 14q3.2 genotype and ploidy. Paving the road for tailored therapies

Myeloma cells from a minority of patients retain some B-cell marker expression, namely CD19 or CD20 but never both. Most CD19$^+$ and CD20$^+$ MM co-express CD27 at diagnosis. CD19 is found in very rare cases, i.e. 2.5% (9 out of 362, Table 1). CD20 expression is more frequent than CD19 expression since around 14% of patients express CD20 at diagnosis (29 out of 209).[13] Of note, CD20 is expressed by two HMCL out of 29 (Table 3). CD19 or CD20 expression is clearly associated with the morphology of small mature plasma cells and the t(11;14) translocation (*unpublished data*).[13] The expression of CD19 and CD20 is mutually exclusive in myeloma cells, unlike in Waldenström cells, where they are co-expressed. As for phenotype and genotype correlation, it appears that CD19$^+$ or CD20$^+$ MM represents a first entity characterized by more frequent t(11,14) and diploidy. Conversely, patients with CD19$^-$CD20$^-$CD27$^-$ MM are characterized by frequent t(4;14) or t(14;16) and non-hyper diploidy; these myelomas represent another entity with intermediate or even plasmablastic morphology and a more adverse prognosis.[7,59] A third entity of MM is characterized by the expression of CD117 (and a lack of both CD19 and CD20) and a hyper diploidy (without recurrent 14q32 translocations). The expression of CD117 is associated with a more favorable outcome.[43] Indeed, we observed that the expression of CD117 seems to be restricted to patients with indolent MM (*manuscript in preparation*). Furthermore, its expression is significantly less frequent in relapse and in HMCL[3,10,59] (*personal data*).

Therapy targets for all MM and different subsets or entities of MM

Based on phenotypes, we should distinguish antibody-based therapies for all patients from therapies for particular entities or subpopulations of MM patients. On the other hand, phenotype also opens up the possibility of apoptosis-based therapies as illustrated with CD45.

CD138, more than CD38, appears to be a particularly attractive target for antibody-based therapy of all MM.[12,15,17] Indeed, all myeloma cells from all patients express CD138 and the level of its expression is higher in myeloma cells than in normal PC. CD38 is also expressed by all myeloma cells (absent in only very rare cases). However, CD38 is more broadly expressed than CD138 and the level of expression in myeloma cells is lower than in PC. Another antibody-based therapy for all MM patients could target LFA-1/CD45 since their co-expression is restricted to the more proliferating compartment.[51]

On the other hand, different surface molecules could be targeted as individual therapies for either well-defined MM entities i.e., CD19, CD20, CD27 or CD117, or subpopulations of MM i.e., CD33, CD52.[7,10-11,13-14,16,58] Clinical grade monoclonal antibodies exist for CD20, CD33 and CD52 and clinical trials are ongoing for some of them.

CD45 expression clearly controls myeloma cell response to IL-6 and IGF-1, the two major growth factors for myeloma cells. It appears clear that CD45 expression is a critical determinant for either anti-IL-6R or anti-IGF1R therapeutic approaches in MM.

Concluding remarks

Establishing the phenotype of myeloma cells and differentiating it from that of normal PC has proven very useful for the identification and characterization of myeloma cells, including intraclonal subpopulations. Phenotype studies have also enabled the isolation of pure myeloma cells, a purification required for numerous studies, e.g. production of growth factors, response to growth factors, identification of genetic alterations. The comparison of patients' phenotypes has identified markers for prognosis as well as markers for tailored targeted therapies. Finally, phenotype studies have triggered the emergence of a myeloma cell growth model, which is the first step towards identifying myeloma progenitor cells. We stress that the phenotype approach will be the prime way to hunt for myeloma stem cells. In the immediate future, the phenotype should be a criterion for myeloma diagnosis, for the follow-up of patients with monoclonal gammopathy of undetermined significance in anticipation of MM transformation, for minimal residual disease monitoring, for treatment response evaluation and for early detection of relapse.

RB and CPD wrote the paper; JLH and PM are the physicians involved in MM treatment; GJ, NR, SBN and MA were involved in previously published original research reviewed in this article

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