Anti-CD20 Therapy Acts via FcγRIIIA to Diminish Responsiveness of Human Natural Killer Cells

Cristina Capuano¹, Maddalena Romanelli², Chiara Pighi², Giuseppe Cimino³,⁴, Angela Rago⁴, Rosa Molfetta², Rossella Paolini², Angela Santoni², and Ricciarda Galandrini¹

Abstract

Natural killer (NK) immune cells mediate antibody-dependent cellular cytotoxicity (ADCC) by aggregating FcγRIIIA/CD16, contributing significantly to the therapeutic effect of CD20 monoclonal antibodies (mAb). In this study, we show that CD16 ligation on primary human NK cells by the anti-CD20 mAb rituximab or ofatumumab stably impairs the spontaneous cytotoxic response attributable to cross-tolerance of several unrelated NK-activating receptors (including NKGD2, DNAM-1, NKP46, and 2B4). Similar effects were obtained from NK cells isolated from patients with chronic lymphocytic leukemia in an autologous setting. NK cells rendered hyporesponsive in this manner were deficient in the ability of these cross-tolerized receptors to phosphorylate effector signaling molecules critical for NK cytotoxicity, including SLP-76, PLCγ2, and Vav1. These effects were associated with long-lasting recruitment of the tyrosine phosphatase SHP-1 to the CD16 receptor complex. Notably, pharmacologic inhibition of SHP-1 with sodium stibogluconate counteracted CD20 mAb-induced NK hyporesponsiveness, unveiling an unrecognized role for CD16 as a bifunctional receptor capable of engendering long-lasting NK cell inhibitory signals. Our work defines a novel mechanism of immune exhaustion induced by CD20 mAb in human NK cells, with potentially negative implications in CD20 mAb-treated patients where NK cells are partly responsible for clinical efficacy.

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cytotoxic response due to the cross-tolerance of several unrelated NK-activating receptors, without affecting the ability to secrete IFNγ. Importantly, analogous observations were obtained in NK cells from patients with CLL in an autologous setting. We also provide evidence that in hyporesponsive NK cells, stimulation of the cross-inhibited receptors results in the defective tyrosine phosphorylation of signaling elements critical for NK cytotoxicity such as SLP-76, PLCγ2, and Vav1, likely imputable to the long-lasting recruitment of SHP-1 to CD16 receptor complex.

Materials and Methods

Patients and healthy donors

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors of Transfusion Center of Sapienza University (Rome, Italy) or from patients with CLL of Hematology Unit, S. Maria Goretti Hospital (Latina, Italy). The study was conducted according to protocols approved by our local institutional review board and in accordance with Declaration of Helsinki. Written informed consent was obtained from all patients. The diagnosis of CLL was based on criteria recommended by the International Workshop on Chronic Lymphocytic Leukemia; the stage of disease was assessed according to the Rai staging system (20). In all specimens, the percentage of CD5+CD19+ CLL cells was more than 80%. From each patient, a part of PBMC was used to obtain primary cultured NK cells (see below) and a portion was cryopreserved and stored at −160°C. The day before the experiment, samples were thawed, cultured overnight in complete medium, and then checked for cell viability by trypan blue staining. Only samples with viability more than 90% were used.

Cell systems

Primary cultured human NK cells were obtained from healthy donors or from CLL patients as previously described (21). The experiments were performed on NK cells (CD3−CD56+) more than 80% pure. When specified, NK cells were pretreated for 48 hours with recombinant human IL2 (200 U/mL; R&D Systems).

The following cell lines were used: the human CD20+ lymphoblastoid, Raji, provided by Dr. F.D. Batista (Cancer Research UK, London, UK); the thymic lymphoblastic leukemia, HPB-ALL, obtained from Dr. G. Scala (University “Magna Graecia” of Catanzaro, Catanzaro, Italy; ref. 22); the murine thymoma, BW5147, obtained from A. Moretta (University of Genoa, Genoa, Italy; ref. 23); human lymphoblastoid Daudi and 721.221, T-cell leukemia MOLT-4, erythroleukemia K562, and colon carcinoma CaCo2 were all obtained from ATCC. NK-resistant targets murine pro-B-cell line Ba/F3 and Ba/F3-MICA (stably expressing MICA’0019) cells were provided by Dr. L.L. Lanier (University of San Francisco, San Francisco, CA; ref. 24); Ba/F3-PVR, Ba/F3-CD48 transfectants were kindly provided by Dr. A. Soriani and Dr. A. Zingoni (Sapienza University). Cells were authenticated (last testing January–May 2014) by morphology, growth, and immunophenotypic characteristics, biologic behavior according to the provider recommendations, and tested for mycoplasma contamination by EZ-PCR Mycoplasma Test Kit (Biological Industries). All cell lines were and kept in culture for less than two consecutive months.

Anti-CD20−mediated NK cell stimulation

Raji cells or primary CLL were loaded with 10 μg/mL of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) for 30 minutes at room temperature. Cells were then opsonized with rituximab or ofatumumab conjugates is shown. Bar, 5 μm.

Figure 1.

Rituximab (RTX)- or ofatumumab (OFA)-opsonized target interaction induces CD16 downmodulation at comparable levels. A, IL2-activated NK cells were cocultured for 90 minutes with rituximab (red) or ofatumumab (green)-opsonized or not opsonized targets (blue) and CD16 surface expression was evaluated by immunofluorescence and FACS analysis gating on CD56+ population. One representative of five independent experiments is shown. Gray histogram represents isotype control mAb. B, IL2-activated NK cells were cocultured for the indicated times with rituximab or ofatumumab-opsonized or not opsonized targets (ctrl), stained, and analyzed as in A. CD16 expression was calculated as follows: [mean fluorescence intensity (MFI) of the stimulated samples/MFI of not stimulated sample] × 100. Data from three independent experiments (mean ± SEM) are shown. C, IL2-activated NK cells were cocultured for 60 minutes with rituximab (NK/Raji-RTX) or ofatumumab (NK/Raji-OFA)-opsonized targets. Fixed and permeabilized cells were stained with GAH and anti-CD16 mAb. A representative image of conjugates (n = 55) or isolated NK or Raji cells is shown. The overlap of merged and differential interference contrast (DIC) images of conjugates is shown. Bar, 5 μm.
Primary cultured NK cells were mixed [effector:target (E:T) = 2:1] with targets, briefly pelleted, and cocultured for 90 minutes or different times, where indicated. NK cells were then recovered by negative selection on streptavidin-coated Dynabeads (Invitrogen, Life Technologies). When specified, immunomagnetically isolated NK populations were replated in culture medium, supplemented or not with IL2 (200 U/mL).

Evaluation of cytotoxic activity and IFNγ release
To analyze cytotoxic activity, rituximab- or ofatumumab-experienced NK populations were assessed in a 4-hour 51Cr release assay. IFNγ was analyzed by commercial ELISA kit (Thermo Fischer Scientific; for details, see Supplementary Data).

Statistical and densitometric analysis
Statistical significance between two groups was determined by 2-tailed, paired Student t test. Differences among multiple groups were analyzed with one-way ANOVA using Prism 5 software (GraphPad). Where indicated, values were expressed as mean ± SEM and differences were considered to be statistically significant when P < 0.05. Quantification of specific bands (pixel intensity) was performed with ImageJ1.41o software (NIH, Bethesda, MD).

Additional methods can be found in Supplementary Methods.

Results
NK cell interaction with anti-CD20–opsonized targets induces CD16 internalization
To compare the kinetics of CD16 downmodulation induced by two class I anti-CD20 mAbs, we evaluated CD16 levels in primary human NK cells upon interaction with rituximab- or ofatumumab-opsonized CD20+ targets. Hereafter, in all experiments, we used NK cells stimulated with not opsonized target as control population. Our data (Fig. 1A and B) show a progressive CD16 downmodulation that was superimposable in NK cells interacting with rituximab- or ofatumumab-opsonized targets. CD16 expression reached minimum levels (~40% of control) in 2 hours of interaction, keeping a plateau of downmodulation until 18-hour stimulation (Fig. 1B).

Because both internalization and/or receptor shedding may contribute to anti-CD20–induced CD16 reduction (25), we evaluated CD16 dynamics by fluorescence microscopy (Fig. 1C). As compared with the ring pattern of unconjugated cells, CD16 distributed in intracellular dots in NK cells interacting with rituximab- or ofatumumab-opsonized targets, thus indicating an internalization process. No evidence of anti-CD20 internalization was observed; instead, it appeared capped at cell membrane, as reported by others (26), in some extent colocalizing with residual surface CD16. Furthermore, by comparing CD16 levels in fixed and fixed/permeabilized NK cells, we observed that almost 50% of downmodulated CD16 was the result of internalization occurring during opsonized target interaction (Supplementary Fig. S1).

These data demonstrate that the interaction with rituximab- or ofatumumab-opsonized targets similarly induces surface CD16 downmodulation, which is partially attributable to receptor internalization.

NK cell interaction with anti-CD20–opsonized targets results in a marked defect of cytotoxic responses
We investigated the impact of the NK cell interaction with opsonized targets on their cytolytic potential. Hereafter, “experienced NK cells” define primary cultured NK cells immunomagnetically isolated upon 90 minutes of coculture with rituximab- or ofatumumab-opsonized targets. Cytotoxic activity of anti-CD20 experienced NK cells was assayed against rituximab-opsonized or -sensitive targets to test ADCC or spontaneous cytotoxicity, respectively. As expected, experienced NK cells exhibit a reduced ability to execute ADCC (Fig. 2A), but, more interestingly, we observed a marked impairment of the cytotoxic activity toward a panel of sensitive targets, that was comparable in rituximab- or ofatumumab-experienced NK cells (Fig. 2A).

Because in our experiments the impact of rituximab or ofatumumab on NK cell responsiveness was largely superimposable, we focused our analysis on rituximab.

The analysis of the kinetics of anti-CD20–dependent hyporesponsiveness revealed that it was already evident after 30 minutes and reached a plateau between 90 and 180 minutes of interaction with opsonized targets (Fig. 2B). We then addressed the persistence of the hyporesponsiveness by testing the cytotoxic activity of NK cells maintained in culture for different lengths of time upon opsonized target detachment. Albeit a partial rescue was observed, a clear reduction of the spontaneous cytotoxicity persisted until 48 hours. Notably, when the same effector population was preactivated and cultured in the presence of IL2, a progressive recovery of the cytolytic function that paralleled CD16 re-expression was evident, being almost complete after 48 hours (Fig. 2C).

These experiments demonstrate that CD16 downregulation induced by rituximab- or ofatumumab-opsonized target interaction is associated with a persistent defect of the cytotoxic responses.

NK hyporesponsiveness involves the cross-tolerance of different activating receptor systems
With the aim to investigate, at individual receptor level, whether anti-CD20–induced NK cell hyporesponsiveness could regard selected activating receptors, we tested the ability of rituximab-experienced NK cells to kill resistant targets (Supplementary Fig. S2A) bearing individual receptor ligand, that is, MHC-I-related chain A (MIC-A), poliovirus receptor (PVR), or CD48, to explore NK2CD10, DNAM-1, or 2B4-dependent lysis, respectively; moreover, we took advantage of BW5147 thymoma cell line whose killing is mostly due to NKp46 triggering (Supplementary Fig. S2B). Our findings show a significant defect of the cytotoxic activity triggered by the different receptors analyzed demonstrating that rituximab-mediated CD16 aggregation cross-inhibits multiple and unrelated activating receptor families (Fig. 2D, Supplementary Fig. S2C).

To assess whether the defect of spontaneous cytotoxicity could be related to the downregulation of activating receptors occurring during target interaction, we analyzed the expression levels of a panel of activating and adhesion receptors. Unlike CD16 expression, we did not observe significant differences in rituximab-experienced NK cells with respect to control population or to the NK cells that did not interact with target cells (unstimulated; Fig. 2E). When we evaluated perforin and granzyme B, we observed that cytolytic mediator levels in
rituximab-experienced NK cells was similar to that of control populations, leading us to exclude that the cytotoxic defect depends on the depletion of lytic mediators (Fig. 2F). Analogously, the same rituximab-induced hyporesponsive phenotype was also observed in the absence of IL2 stimulation (Supplementary Fig. S3).

Rituximab-opsonized primary B-CLL cells tune-down CD16 expression and cytolytic potential in autologous NK cells

To extend our observations to NK cells of patients potentially candidate to rituximab treatment, we analyzed 6 untreated patients with CLL (Supplementary Table S1). Primary NK cells were opsonized with rituximab and allowed to interact with autologous primary NK cells. Rituximab-opsonized NK cells were tested for CD16 expression and for spontaneous cytotoxicity. Our findings show that NK cell interaction with autologous-rituximab-opsonized leukemia cells induces a marked downmodulation of CD16 receptor (Fig. 3A) that is associated with a relevant defect of spontaneous cytotoxic activity against Raji cells and PVR-expressing target cells (Fig. 3B and C).

Lytic granule secretion but not IFNγ production is impaired in rituximab-experienced NK cells

We sought to investigate whether the defective cytotoxic activity could selectively involve different steps of the lytic event (27, 28). Our data show that neither the ability to form conjugates with targets nor the polarization of lytic granules at cytolytic synapse (Fig. 4A and B) resulted impaired. However, when we measured the ability to secrete lytic granules in response to target stimulation by assessing CD107a surface levels, we observed a significant defect of lytic granule exocytosis in rituximab-experienced NK cells with respect to unstimulated cells. Such impairment was observed in both CD16+ and CD16 downregulated populations (Supplementary Fig. S4). In line with our evidences of a normal lytic granule content in hyporesponsive cells, degranulation induced by phorbol 12-myristate 13-acetate (PMA) plus ionomycin resulted unaffected (Fig. 4C).

We then explored the ability of rituximab-experienced NK cells to secrete IFNγ. We observed that NK cell stimulation with target cells, with mAbs specific for the cross-inhibited receptors or with IL2, induced a comparable amount of IFNγ with respect to control populations (Fig. 4D).

These data demonstrate that the hyporesponsive status involves a selective defect of lytic granule exocytosis but not the ability to produce IFNγ.

Defect of SLP-76, PLCγ2, and Vav1 tyrosine phosphorylation in rituximab-experienced NK cells

Assuming that CD16 downregulation may subtract dose-limiting signaling elements (29), we analyzed the phosphorylation events downstream to the cross-inhibited receptors. On the basis of our observation of a defective lytic granule exocytosis, we focused our analysis on signaling pathways controlling granule secretion at the cytolytic synapse. Our data demonstrate a marked defect on tyrosine phosphorylation of SLP-76 at Y1217 residue, induced by CD16 itself or by NKp46, NK2D, or DNAM-1 stimulation in rituximab-experienced NK cells. Concomitantly, we also observed reduced levels of PLCγ2 tyrosine phosphorylation at Y1217 residue. Similarly, receptor-induced Vav1 tyrosine phosphorylation at Y160 residue was also reduced, although some basal levels of tyrosine phosphorylation were evident in rituximab-experienced cells (Fig. 5A and B). Conversely, in hyporesponsive cells, Akt and Erk phosphorylation downstream cross-inhibited receptors resulted unaffected (Fig. 5C).

These results demonstrate that in rituximab-experienced NK cells tyrosine phosphorylation of SLP-76, PLCγ2, and Vav1, downstream to activating receptors is impaired.

Recruitment of SHP-1 tyrosine phosphatase to CD16 receptor complex upon rituximab stimulation

The defective tyrosine phosphorylation of SLP-76, PLCγ2, and Vav1 led us to hypothesize the involvement of a tyrosine phosphatase coupled to CD16. We sought to analyze whether CD16 experiences NK cells were opsonized with rituximab and allowed to interact with autologous NK cells. Our findings show that NK cell interaction with autologous-rituximab-opsonized leukemia cells induces a marked downmodulation of CD16 receptor (Fig. 3A) that is associated with a relevant defect of spontaneous cytotoxic activity against Raji cells and PVR-expressing target cells (Fig. 3B and C).

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was maximally phosphorylated, but appeared upon 30 minutes, further increasing at 60 minutes of rituximab stimulation (Fig. 6A). In contrast, we did not observe any evidences of SHP-2 recruitment. Intriguingly, the aggregation of CD16 obtained with anti-CD16 (B73.1) mAb did not induce a detectable SHP-1 recruitment. Of note, in such aggregation conditions, although a very efficient CD16 downmodulation occurred (Fig. 6B), NK cells preserved their cytolytic potential (Fig. 6C).

These experiments show that rituximab-mediated but not anti-CD16 mAb-mediated stimulation induces the recruitment of SHP-1 to CD16 receptor complex.

Discussion

The central observation of our report is that preligation of CD16 by anti-CD20 mAbs reduces the ability of NK cells to kill target cells; indeed, we demonstrate that the outcome of NK cell interaction with rituximab- or ofatumumab-opsonized targets is a comprehensive reduction of both Ab-dependent and -independent cytotoxic potential. Such NK hyporesponsive status is almost fully established upon 30 minutes of CD16 ligation and persists at least until 48 hours of stimulation. Our data highlight that the hyporesponsiveness does not involve the ability to secrete IFN-γ in response to the cross-inhibited receptors. This observation is not really surprising, taking into account the uncoupling of the signaling pathways and molecular machinery leading to the activation of the cytolytic program or to the production of cytokines, including IFN-γ (33).

The impairment of ADCC response can easily be explained by CD16 downmodulation induced by the stimulation of NK cells with anti-CD20–opsonized targets, which is only partially attributable to receptor internalization; such finding complements recent observations demonstrating that rituximab stimulation, through activation of metalloprotease-17 (ADAM17), promotes CD16 shedding (25).
Less expected is the finding that anti-CD20–mediated CD16 stimulation also promotes a marked reduction of the ability of NK cells to kill sensitive targets attributable to the cross-inhibition of multiple unrelated activating receptors, including NKG2D, 2B4, NKp46, and DNAM-1, which are coupled to both ITAM- and non–ITAM-containing signaling adaptors (14, 15). The observation that preligation of CD16 induces NK cell hyporesponsiveness is strengthened by the finding that the exposure of primary NK cells from patients with CLL to rituximab-opsonized autologous leukemia cells induced a significant reduction of natural killing potential. Importantly, our observations might account for the marked defect of spontaneous cytotoxicity in ex vivo isolated NK cells from patients with diffuse large B-cell lymphoma treated with rituximab (34). Furthermore, the cooperation of activating receptors, stimulated by novel therapeutic mAbs or by tumor ligands, in determining the overall response to tumor targeting mAbs have been recently highlighted (35–37); their cross-tolerance induced by rituximab treatment may negatively affect the clinical outcome.

We also provide evidences that the cytotoxic defect is not attributable to an altered phenotype or to depletion of cytolytic mediators: indeed, the levels of activating and adhesion receptors as well as of perforin and granzyme B are largely unaffected.

We were much intrigued in understanding the molecular basis of NK hyporesponsiveness induced by CD16 preligation. In natural killing, a mechanistic role of CD16 has been defined through the demonstration that CD16-dependent proximal signaling elements may be exploited by other activating receptors (29). We explored the possibility that the persistent engagement of CD16 may lead to the inhibition of critical molecules acting in signal integration downstream to activating receptors. On this regard, the adaptor protein SLP-76 has been shown to orchestrate the formation of a molecular platform allowing the tyrosine phosphorylation of...
critical signaling elements such Vav1 and PLCγ2 (14, 38). Our data show in hyporesponsive NK cells a marked defect in SLP-76, PLCγ2, and Vav1 tyrosine phosphorylation induced by the stimulation of cross-inhibited receptors or by CD16 itself. Furthermore, we observed that the activation of PI3K and MAPK-dependent signals resulted unaffected. These data, in the context of our observation of a selective defect of lytic granule exocytosis with normal granule polarization in rituximab-experienced NK cells, recapitulate the dichotomy of molecular signals governing polarization versus degranulation: the pathway sequentially involving PI3K/Rac1/Pak1/MEK/ERK1/2 has been referred as pivotal for granule polarization, whereas PLCγ/calcium-dependent signals are the hallmark for their secretion (27, 28, 39).

![Figure 5](image)

Figure 5. Defect of SLP-76, PLCγ2, and Vav1 tyrosine phosphorylation in rituximab (RTX)-experienced NK cells. NK cells were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. Recovered NK cells were stimulated with anti-CD16, anti-NKG2D, anti-DNAM-1, anti-NKp46, or isotype-matched anti-CD56 mAb (ctrl mAb) followed by GAM F(ab)2. An equal amount of proteins was immunoblotted with Abs anti-phosphorylated proteins of the indicated specificity. The same membranes were reprobed as indicated for sample normalization. For each phosphorylated protein, control and rituximab membranes were derived from the same film. One representative experiment of three performed is shown. B, data from three independent experiments as in A are presented as min-to-max bar graphs with average mean lines. ***, P < 0.0005; **, P < 0.01; *, P < 0.05 paired Student t test. C, NK cell lysates were immunoblotted with the indicated Abs. The numbers between lanes represent phosphorylation levels of the indicated proteins after normalization with the relative total levels.
We firstly provide evidences of the involvement of SHP-1 in CD16-dependent cross-tolerance of activating receptors. Indeed, we observe that rituximab stimulation promotes the recruitment of SHP-1 phosphatase, but not SHP-2, to the tyrosine phosphorylated CD16 \(\zeta\) chain. This recruitment was delayed (30 minutes) and long-lasting (1 hour) with a time course drastically different from the classical rapid recruitment of SHP-1 by ITIM-containing MHCI inhibitory receptors in condition of co-aggregation with activating receptors (32).

We propose that the recruitment of SHP-1 to targeted CD16 promotes its accumulation at the plasma membrane, reaching a...
threshold level that would allow the dephosphorylation of signaling proteins, thus desensitizing activating receptors subsequently stimulated (Figure 7). Indeed, both SLP-76 and Vav1 have been shown to be key targets of SHP-1 triggered by killer inhibitory receptors in NK cells (40, 41). We are currently investigating by imaging approaches the spatial coordination of inhibitory signals; on this purpose, a recent report demonstrated a raft-dependent co-segregation of SHP-1 with signaling effectors into intracellular clusters (called inhibisomes), providing an appropriate scaffold for substrates interaction during inhibitory process (42). A functional role of SHP-1 in mediating CD16-dependent hyporesponsiveness is indicated by our observation that SHP-1 pharmacologic inhibition with SSG, known to inhibit SHP-1 (30), significantly interferes with rituximab-induced inhibitory responses.

On the basis of our data showing a long-lasting NK exhaustion following rituximab stimulation, we cannot exclude that a transcriptional or a posttranscriptional modulation may contribute to NK hyporesponsiveness. Accordingly, the recovery of NK responsiveness driven by IL2 treatment, strictly associated to the restoration of CD16 levels, may indicate a de novo gene expression and protein synthesis. Indeed, recent data demonstrated that the calibration of NK cell reactivity is subjected to a genetic-based tuning attributable to transcription factor modulation (19, 43).

Strikingly and in contrast with rituximab, receptor ligation by anti-CD16 mAb was ineffective in promoting SHP-1 recruitment. Notably, in the same stimulation conditions, the ability of NK cells to kill sensitive targets was preserved, indicating a relationship between aggregation conditions and the ability to promote inhibitory signal toward heterologous receptors. It would be interesting to address whether other therapeutic mAbs, acting through the triggering of ADCC, such as trastuzumab and cetuximab or with improved affinity for CD16, as obinutuzumab, may also mediate a CD16-dependent inhibitory signal.

Overall our findings are in line with the emerging paradigm considering that the targeting of some classically activating Fc receptors in myeloid cells with low-avidity ligands, paradoxically transmit inhibitory signals, through the recruitment of SHP-1, leading to the inhibition of a number of innate immune responses (44–46).

We identify here a mechanism of exhaustion of NK cells likely contributing to the resistance and/or to the development of a refractory status to rituximab-containing regimens leading to disease relapse or progression (6). Furthermore, our data may provide insights on the high frequency of viral reactivation in rituximab-treated patients, which may implicate an NK hyporesponsiveness leading to reduced immunosurveillance (47).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Capuano, R. Galandrini
Development of methodology: C. Capuano, R. Molfetta
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Capuano, M. Romanelli, C. Pighi, G. Cimino, A. Rago, R. Molfetta
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Capuano, R. Paolini, A. Santoni, R. Galandrini
Writing, review, and/or revision of the manuscript: C. Capuano, G. Cimino, R. Paolini, A. Santoni, R. Galandrini
Study supervision: R. Galandrini

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