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Cellular Interferon Gamma and Granzyme B Responses to Cytomegalovirus-pp65 and Influenza N1 Are Positively Associated in Elderly

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Abstract

Morbidity and mortality in the elderly are associated with viral infections, including influenza and cytomegalovirus (CMV). With increasing age, cellular immunity gains importance in protection to influenza, but latent CMV is highly prevalent and associated with immune dysfunction. An insight into the association between immunity against influenza and CMV adds to the understanding of healthy aging. Here we first aimed to measure influenza-specific cellular immunity using granzyme B (GrzB) and interferon-gamma (IFN-γ)-ELISpot. Next, influenza-specific cellular immunity was associated with humoral and cellular CMV-specific immunity in healthy 65+ elderly. Vaccine trial participants gave additional blood samples 3 weeks after receiving a H1N1 containing vaccine. CMV serology was determined and peripheral blood mononuclear cells were stimulated with influenza N1 or CMV pp65-derived peptide pools for 7 days and rechallenged to assess antigen-specific GgrzB and IFN-γ responses using ELISpot assays. Results were compared using chi-square and correlation analysis. Eighty-three individuals (60% men, 65% CMV IgG+, age range 65–78y) participated. We found significant positive associations between IFN-γ and GrzB responses to both influenza and CMV, but also between a positive CMV serostatus and an influenza N1-specific activation marker response ($p = 0.013$). CMV pp65 responses were detected in CMV IgG+ individuals, but remarkably also in CMV IgG- individuals (27%). In this study, following influenza vaccination, elderly with cellular immunity against CMV were more likely to have cellular immunity against influenza vaccine N1 antigen. These findings stress the need to continue exploring the possible role of CMV in immunosenescence.

Introduction

Influenza and cytomegalovirus (CMV) infection have both been associated with morbidity and mortality in the elderly; the first during its acute attacks and the second during its lifelong latent presence (13,24,29,35,47).

Seasonal influenza virus epidemics cause half a million deaths annually worldwide and remain one of the largest global threats to human health, especially in vulnerable elderly (46).Clinical influenza vaccine effectiveness is lower in elderly and less predictable by humoral immune responses than at a younger age (13). The importance of T-cell responses as second-line defense against influenza disease and complications increases with age (35). Recent studies in humans suggest that in elderly, T-cell responses to internal proteins of influenza virus may be better correlates of protection to influenza than influenza antibodies (37,43).

Latent CMV infection is highly prevalent in elderly, with a reported prevalence of 70–90% in individuals older than 50 years and >90% in individuals older than 65 years in industrialized countries (2,20,40). Persistent CMV infection has been associated with immune dysfunction and increased morbidity and mortality, in immunocompromised patients and also in the elderly, depending on the virus–host balance (28,29). Chronic inflammation is hypothesized as a key pathophysiological process through which CMV infection contributes to adverse health outcomes in older adults (3,5,24,29,35,42). CMV could thus be an important threat for the health of the growing population of elderly. The presence of IgG antibodies against CMV (CMV IgG) is

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generally considered the main marker of infection, which is considered to be latent in cells of the myeloid lineage, with mostly asymptomatic reactivations during which the virus replicates in differentiated cells, occurring at unknown intervals (5). CMV IgG seropositivity is associated with specific alterations in both the $CD8^+$ and $CD4^+$ T-cell compartments, which are most obvious at older age. (34) It is not clear if and how these alterations do impact the capacity of T cells to effectively respond to infections. The capacity to respond to vaccine antigens is a fitness indicator for the adaptive immune system and has, as such, been studied in relationship with CMV IgG serology, with diverging results. CMV IgG seropositivity has been associated with a decreased humoral response to influenza vaccination in the elderly in some studies (10,24,39), whereas others could not confirm such an association (7) or found it only in healthy adults younger than 65 years and not in elderly (41). Association with cellular immunity to influenza has not yet been studied extensively, but CMV seropositivity was recently found to be associated with decreased $CD4^+$ T-cell responses against influenza A core proteins (9).

To gain insight into the association between immunity against CMV and influenza in elderly, we assessed CMV immunity and cellular responses against influenza N1 antigen in a cohort of 65+ who recently received H1N1 containing vaccine.

Methods

Study population and samples

Community-dwelling elderly in stable health condition were recruited for an industry-sponsored trial with a trivalent split-virion influenza vaccine at the Center for the Evaluation of Vaccination, University of Antwerp, during two periods in the 2011–2012 winter season (the first in November-December 2011 and the second in March 2012). Trial participants were invited to give additional serum and heparinized blood samples (40 mL) in a consecutive investigator-driven study within 3 weeks after vaccination, after having completed all trial visits, and after being informed of the aims of the study, and signing a separate informed consent. The study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Antwerp University Hospital.

Participating elderly ($n = 83$) were 65–78 (mean 69) years of age, 60% of them were male and 57% had a history of prior influenza vaccination but not in the preceding 2010–2011 season. None of them had been vaccinated with the pandemic A (H1N1) influenza vaccine. Serum and peripheral blood mononuclear cells (PBMC) were isolated and frozen within 24 h after collection. PBMC were isolated by standard Ficoll-Hypaque Plus density gradient centrifugation (GE Healthcare) from freshly obtained heparinized blood and frozen in 90% fetal bovine serum (FBS) (Life Technologies; 10270–106) supplemented with 10% DMSO (Sigma-Aldrich; D2650). Filled cryovials were put into Nalgene cryoboxes before freezing at −80°C. Afterward the cells were stored in liquid nitrogen. Serum samples were frozen at −40°C after centrifugation within 4 h after collection, until batch analysis.

CMV virus serology

IgG directed against CMV pp150, pp28, p38, and p52, and IgM against CMV pp150 and p52 were determined with ELISA on thawed serum samples (Roche COBAS). According to the manufacturer's instructions, samples were considered seropositive if ≥1 EU/mL anti-CMV IgG was detected.

ELISpot assay

The following virus-derived peptide pools were used for stimulation of antigen-specific T cells: (i) Neuraminidase A/California/08/2009 (H1N1) Pepmix (JPT Peptide Technologies GmbH, PM-INFA_NA, C7FH14), derived from the same H1N1 virus strain as present in the vaccine, further referred to as N1 peptide pool; and (ii) HCMV pp65 peptide pool (NIH AIDS Reagent Program, 11549) (17). The N1 antigen, although being a weak cellular antigen, was selected because all participants had received H1N1 containing split-virion vaccine (A/California/7/2009) and should thus have a similar N1 immunity status. For T-cell activation experiments, 1×10^6 cryopreserved PBMC were cultured in the presence of 1 μg/mL of the influenza N1 peptide pool or 1 μg/mL of the CMVpp65 peptide pool. The experiments were performed in T25 tissue culture flasks in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% human AB serum at 37°C in a CO_2-humidified atmosphere. After 7 days of coculture, T cells were analyzed for antigen specificity by determining interferon gamma (IFN-γ) and granzyme B (GrzB) production following antigenic restimulation by means of IFN-γ (Mabtech) and GrzB (Diaclone SAS, Besancon, France) ELISpot, respectively, according to the manufacturer's instructions. The 7-day incubation period was added because the ELISPot, although being a sensitive testing method, did not allow to detect GrzB responses *ex vivo* and was applied for the IFN-γ ELISpot to be consistent in methodology.

For detection of IFN-γ producing antigen-specific activated T cells, PBMC were incubated at a concentration of 1–1.5×10^5 cells/well and rechallenged with either 1 μg/mL of influenza N1 or 0.25 μg/mL CMVpp65 peptide pools in anti-human IFN-γ (Mabtech) antibody-coated 96-well polyvinylidene fluoride (PVDF) plates (Millipore), for at least 16 h and not more than 24 h at 37°C in a CO_2-humidified atmosphere. Nonstimulated PBMC were used as a control and each condition was measured in quadruple. For the detection of spots, a biotin-labeled anti-human IFN-γ (Mabtech) was used. Spots were visualized following incubation with horseradish peroxidase-labeled streptavidin (BD Biosciences; 557630) and AEC chromogen (BD Biosciences, 551951).

GrzB producing antigen-specific activated T cells were detected with a GrzB ELIspot (Diaclone; 856.131.005). PBMC were incubated at a concentration of $1–1.5 \times 10^5$ cells/well and rechallenged either 1 μg/mL of influenza N1 or 0.25 μg/mL CMVpp65 of the respective peptide pools in anti-human GrzB antibody-coated 96-well PVDF plates (Millipore) for at least 16 h and not more than 24 h at 37°C in a CO_2-humidified atmosphere. Nonstimulated PBMC were used as a control and each condition was measured in quadruple. Spots were detected using a biotin-labeled anti-human GrzB and visualized following incubation with alkaline phosphatase-labeled streptavidin and BCIP/NBT chromogen. Frequencies of antigen-specific cytokine secreting cells were calculated based on the number of spots counted using an automated iSpot Reader system (AID GmbH) and analyzed using AID

ELISpot software version 5.0. Mean spot-forming cells (SFC) per 10^6 cells were calculated from the difference of the means of the quadruplicate experimental (stimulated) wells and the quadruplicate control (nonstimulated) wells, per subject. If the signal in the control wells (background signal) exceeded the signal in the experimental wells, the mean SFC was set to zero. Mean SFC ratios (mean stimulated SFC/mean unstimulated SFC) were calculated as well. For the definition of a positive response (at subject level), guidelines from the Cancer Vaccine Consortium were followed: per 10^6 PBMC, the antigen-specific mean SFC must be greater than or equal to 15 spots and the signal in experimental wells at least two times as high as in the control wells (16,23).

Statistical analysis

Results were compared with chi-square, Spearman's rho (correlation), and nonparametric analysis as appropriate using IBM SPSS Statistics 22 and GraphPad Prism 5 software. Postanalysis Bonferroni correction was applied in case of multiple comparisons. For quantitative analysis on SFC, values of nonresponders were included except if the difference between mean stimulated and mean unstimulated SFC was below 15, to correct for high background signal or low overall signal.

Results

Humoral responses

CMV serology was available for all participants, 54 (65.1%) of them were CMV IgG positive, of whom 4 were CMV IgM positive as well.

Cellular responses

PBMC could not be sampled from two participants. For CMVpp65-specific responses, the ELISpot could not be interpreted due to high background signal in 5 and 10 participants for IFN-γ and GrzB, respectively. Among the remainder, according to our response definition, 41 (53.9%) had a positive CMVpp65-specific IFN-γ response, and 31 (42.5%) a positive GrzB response. In the 67 participants of whom ELISpot results for both markers of activation were available, responses were significantly associated with 37.3% double negatives (no IFN-γ and no GrzB response after CMVpp65 stimulation) and 35.8% double positives (chi^2, $p<0.001$). Correlation analysis was done on mean SFC ratios because they are more informative than counts in case of high background signal and because they were much less skewed than mean SFC counts (Table 1). Correlation between mean SFC ratios for IFN-γ and GrzB against CMVpp65 was moderate (Spearman's rho 0.49, $p=0.002$). ◀T1

Influenza N1-specific GrzB ELISpot could not be interpreted due to high background signal in two participants and showed a positive response in 32 study subjects (40.5%), whereas IFN-γ ELISpot response was available for all samples and was positive in 54 study subjects (66.7%). In the 77 participants for whom ELISpot results for both activation markers were available, a positive IFN-γ response after N1 stimulation was significantly associated with positive GrzB ELISpot responses (Chi^2, $p=0.001$) with 26.0% double negatives and 37.7% double positives. Single positives most often (25/28) had an IFN-γ response. Correlation analysis was done on mean SFC ratios for similar reasons as mentioned for CMVpp65 responses. Correlation between quantitative IFN-γ and GrzB responses (mean SFC ratios) against N1 was weak (Spearman's rho 0.34; $p=0.016$).The highest mean SFC counts for CMV and N1 were seen in the youngest participants, but overall mean SFC counts did not correlate with age (Spearman's rho below 0.2 for both activation markers).

CMV immune response status

First, we evaluated if humoral and cellular immune responses against CMV were concordant. We found significant associations between being CMV IgG seropositive and having positive IFN-γ (chi^2, $p<0.001$) and GrzB (chi^2, $p=0.007$) ELISpot responses against CMVpp65. However, we found no significant association between CMV IgG titer and quantitative CMVpp65-specific ELISpot responses (mean SFC ratios). Also, 9 of 28 CMV IgG seronegatives demonstrated a positive IFN-γ and/or GrzB ELISpot response, whereas 6 out of 40 CMV IgG seropositives who had results available for both ELISpots had no CMVpp65-antigen-specific ELISpot response.

Association between CMV immunity and cellular immunity against influenza N1

We looked for any association between CMV immunity (humoral versus cellular) and cellular immunity against influenza in this recently vaccinated population, and found a significant positive association between CMV serostatus and

Table 1. Quantitative IFN-γ and Granzyme B Production After CMVpp65 or Influenza N1 Stimulation

	Mean experiment/mean control				*Mean experiment/mean control*			
	IFN-γ CMV	*IFN-γ flu*	*GrzB CMV*	*GrzB flu*	*IFN-γ CMV*	*IFN-γ flu*	*GrzB CMV*	*GrzB flu*
Minimum	0	0	0	0	1.1	1.0	1.1	1.0
Q1	15.0	45.0	11.3	8.4	1.9	2.5	1.5	1.8
Median	209.2	140.4	161.1	40.6	3.8	6.5	2.7	2.3
Q2	852.5	423.3	781.3	106.4	11.2	12.6	7.4	3.4
Maximum	4048.3	3796.7	6856.7	3003.3	75.4	60.0	42.3	145.0

Distribution of quantitative IFN-γ and Granzyme B ELISpot results, calculated from experimental and control wells as mean differences (in SFC/million cells) or mean ratios. CMV = after CMVpp65 stimulation in experimental wells; flu = after influenza N1 stimulation in experimental wells; Q1 = 25% percentile; Q2 = 75% percentile; GrzB = granzyme B. ◀AU2

CMV, cytomegalovirus; IFN, interferon; SFC, spot-forming cells.

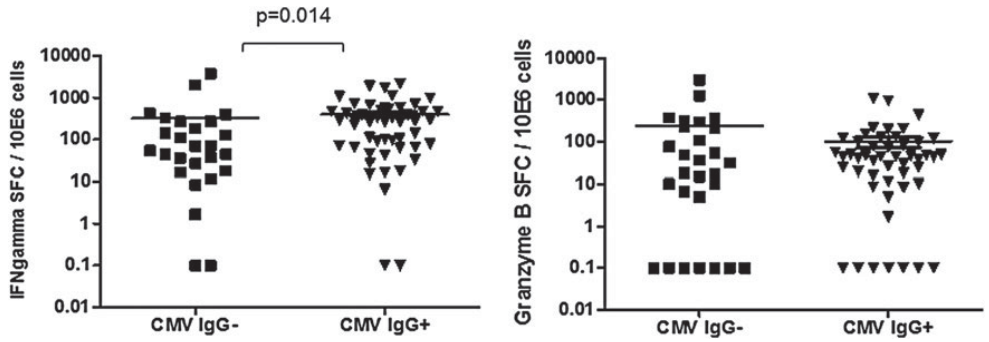

FIG. 1. Mean spot forming cell counts (SFC/10^6 cells) after influenza N1 antigen stimulation, as calculated from the difference of the SFC in the experimental and control wells, measured with IFN-γ (*left hand*) or Granzyme B (*right hand*) ELISpot, compared in CMV IgG seronegative versus seropositive elderly using Mann–Whitney test. CMV, cytomegalovirus; IFN, interferon; SFC, spot-forming cells.

IFN-γ response ($p=0.028$) but not GrzB response on N1 antigen (Fig. 1). A trend to a similar association was seen with CMV Elispot response but significance disappeared after Bonferroni correction ($p=0.09$) (not shown). Also, when we considered production of IFN-γ, GrzB, or both activation markers upon stimulation as having cellular immunity against the antigen, we found both CMV seropositives and subjects having any response (IFN-γ, GrzB, or both) on CMVpp65 stimulation more frequently having a response upon influenza N1 stimulation (Table 2).The difference between subjects with any CMV immunity (cellular or humoral) and those without any such immunity increased in function of N1 response as none, one, or both of GrzB and IFN-γ ELISpots being positive (chi^2 for trends, $p=0.006$).

Also with regard to cellular activation marker responses, we found positive associations between having a GrzB ELISpot response to influenza N1 and to CMVpp65 (Fisher's exact, $p=0.006$), and between IFN-γ response to influenza N1 and to CMV pp65 (Fisher's exact, $p=0.006$). Correlations between mean SFC ratios were weak and not significant: we tested correlation (Pearson) between GrzB ELISpot responses to CMVpp65 and influenza N1 for those who had positive responses for both ($N=18$; $r=-0.15$; $p=0.553$) and between IFN-γ ELISpot responses to CMVpp65 and influenza N1 ($N=31$; $r=0.28$; $p=0.127$).

Discussion

In the current study, we investigated an association between CMV immune status and cell-mediated immune responses against influenza and found an unexpected positive association in healthy elderly people (> 65 years old).

In elderly, cellular immunity as second line of defense is considered important to control influenza infection because humoral responses are often deficient and not allowing sterilizing immunity (13,35). We opted to measure *ex vivo* GrzB production after stimulation with antigen instead of intracytoplasmatic presence because prepacked GrzB can only have an impact *in vivo* if it is effectively released. Effector T cells store perforin and GrzB molecules in their cytolytic granules and on antigen encounter release these molecules toward the target cell. A considerable proportion of T cells in PBMC are GrzB positive due to ongoing stimulation with various environmental antigens, but only recently, activated T cells are capable of exerting perforin/GrzB-mediated cytolytic functions (25). Measuring *ex vivo* activation marker production by ELISpot instead of using a flow cytometric experiment allows for batch analysis in higher numbers and is less expensive.

Using ELISpot, we were able to detect responses against N1, which is a capsular antigen and only induces weak T-cell

TABLE 2. ASSOCIATION BETWEEN HUMORAL AND CELLULAR CMVPP65 IMMUNITY AND CELLULAR IMMUNITY AGAINST INFLUENZA N1, COMBINING RESULTS OF SEPARATE IFN-γ AND GRANZYME B ELISPOT

	No influenza N1 cellular immunity N(%)	*With influenza N1 cellular immunity N(%)*	p*value (Fisher's exact) (total number)*
CMV IgG negative	12 (15.8)	15 (19.7)	0.026
CMV IgG positive	8 (10.5)	41 (53.9)	($N=76$)
No CMVpp65 cellular immunity	12 (18.8)	5 (7.8)	0.006
With CMVpp65 cellular immunity	12 (18.8)	35 (54.7)	($N=64$)

Cellular immunity defined as showing GrzB and/or IFN-γ production (ELISpot) after stimulation with CMVpp65 or influenza N1 antigen, as mentioned. Participants were included if results from all three assays (CMV IgG, IFN-γ ELISpot, GrzB ELISpot) were available, for each mentioned antigen, that is, N1 in the upper rows; CMVpp65 and N1 in the lower rows.

immunity. Studies looking at cellular responses against influenza often used nucleoproteins or matrix proteins (9,37), because they are immunodominant T-cell antigens. Such antigens are, however, not present or only at low level in split and subunit vaccines, which are frequently used, and thus not allowed to make any links with responses induced by such vaccines. For the latter, (subunit) vaccine antigens and strain-specific HA pools have been used successfully by others (6,10,32). Our study demonstrates that cellular response to influenza NA antigen can be detected using IFN-γ and GrzB ELISpot, which could thus be used to assess vaccine response in larger study groups.

Previous studies most often only measured IFN-γ response, which was linked to clinical protection in children vaccinated with life influenza vaccine (11,14). However, only influenza-specific GrzB production has been related to clinical protection from influenza in older adults (21). Feasibility of GrzB ELISpot to detect cellular immunity to influenza was reported earlier (33). In the current study, positive GrzB and IFN-γ responses against the N1 influenza antigen were frequently associated, but a considerable number of IFN-γ responders had no GrzB response, and quantitative responses correlated only weakly. The ELISpot assay used in our study did not allow for differentiation between $CD8^+$ or $CD4^+$ T-cell response, and both cell types might be involved in the measured responses (4). In elderly, both cell types have indeed been shown to be able to produce IFN-γ and GrzB after influenza vaccination (22), although GrzB is primarily produced by activated CD8+ T cells. However, capsular N antigen is a much weaker stimulus for CD8 T cells than for CD4 T cells and it is likely that most of the IFN-γ and GrzB ELISpot responses we found were derived from the latter subset.

We could not detect N1-specific cellular activation marker production in 29.6% of study participants, although they had all been vaccinated with N1-containing influenza vaccine 3 weeks earlier. Similar findings were reported by Salk *et al.*, (33) who used whole H1N1 virus as antigen and were suggested to be due to aging and its negative impact on immunoproliferative responses (8).

We previously reported a negative association between CMV seropositivity and vaccine-induced influenza antibody response in healthy community-dwelling elderly in Belgium participating in a trial with an intradermal influenza vaccine (10). That study also demonstrated an impact of CMV infection on the $CD4^+$ T-cell population with higher frequencies of end-differentiated ($CD28\text{-}CD45^+$) cells in CMV-infected elderly (10). Assessing vaccine response was unfortunately not possible in our study since we lacked prevaccination blood samples and could thus not disentangle memory and vaccine response. Surprisingly, in the current study, CMV-positive status, humoral as well as cellular, was positively associated with having cellular immunity against N1 antigen, with IFN-γ, GrzB, or with both. A positive contribution of CMV to the humoral response to trivalent inactivated influenza vaccine was recently reported by Furman *et al.* but only at a younger age and disappearing in the elderly (12). It might seem odd that the impact of CMV would be different on humoral and cellular influenza immunity, but it was reported earlier that in elderly, humoral and cellular responses against influenza vaccine do not correlate (15,45). Moreover, in case of pre-existing immunity against N1 resulting from N1 strains having circulated in Europe in the 20th century and also from 2010 onward, the vaccine elicited a memory immune response (15,36). The cellular part of this memory response, if any, was obviously not impaired by a CMV-positive status in our study, quite the opposite. This would be consistent with the positive bystander effect of CMV on intercurrent infections that has been postulated by some authors (26,30). $CD4^+$ T-cell responses against mycobacterial antigens were recently reported to correlate with $CD4^+$ T-cell responses against CMV in elderly (38). Derhovanessian *et al.* found CMV-seropositive elderly less likely to have a $CD4^+$ T-cell response to influenza A core protein antigens (9), but the study methodology was not at all comparable to ours and its participants had not been recently vaccinated against influenza.

Finding CMV-seropositive community-dwelling healthy elderly more capable to mount a cellular response against influenza N1 antigen than CMV-seronegative ones could indicate that the observed association between latent CMV infection and severe influenza outcome in elderly would be more related to their frailty status (which is also CMV associated) (1) than to a CMV-related lack of influenza infection control.

Another remarkable finding was that in 9 of 28 CMV-seronegatives, CMV-specific cellular activation marker production could be demonstrated and suggests a history of CMV infection. Seronegative status was confirmed by retesting in all nine subjects. The in-house ELISpot protocol for IFN-γ was highly optimized and validated in former research, and for GrzB the manufacturer's protocol was strictly followed to minimize test errors. Bias due to a specific stimulation is unlikely with only 7 days of culture and no growth factors added, and was anyway corrected for by using quadruplicate control wells for every experiment. In agreement with similar findings previously reported for CMV (19,27) and for hepatitis C (31), our findings thus indicate that the history of CMV infection cannot be ruled out from negative serology.

In the current study, cellular testing was limited to two activation markers produced against one capsular influenza and one CMV antigen, which do not comprehensively reflect the entire cellular response against both viruses. Influenza N1 is not the most potent antigen to induce GrzB responses, as our results confirm. Other studies on influenza cellular immunity have used different antigens and generally no 7-day incubation period before the ELISpot assay, which might hamper comparisons. However, our results are interesting and offer informative guidance for future research initiatives. Using several capsular antigens from different influenza strains next to less strain-specific immunodominant core antigens might strengthen the results of future studies on cellular immunity against influenza. In contrast, CMVpp65 is a highly conserved antigen among CMV strains and is immunodominant for the T-cell response irrespective of major histocompatibility complex haplotype of the host (18,44). Still, demonstration of cellular immunity is only part of the picture in the CMV host–pathogen balance, and information on the activity of the latent virus, for example, by PCR assessment would add value to future research.

The issue of the role of CMV in influenza immunity and vaccine response is a very complex one, while indeed this study, such as many others, addresses only a very specific aspect of the matter. It should not be a surprise that zooming

in at tiny aspects separately may lead to apparently contradictory results, but we believe that integration of the results of studies focusing on different aspects eventually leads to a better understanding of the matter and guides future research.

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Author Disclosure Statement

None of the authors has financial interests in the products used for this study or in the companies producing them.

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