Chapter

A Role for the Measles Virus in Oncology

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Abstract

An association between the measles virus (MV) and classic Hodgkin lymphoma (CHL) was reported by our center in Beer-Sheva, starting in 2003. Our basic evidence on the relationship had been established by immunohistochemistry, using several commercial and specific experimental anti-measles antibodies in about 50% of our patients. It relied also on the reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH) of MV RNA, and overall, was essentially descriptive. The first and only response to our thesis was advanced in 2007 in doublet articles, in a single issue of a Cancer Journal. Our conclusions were hereby dismissed. Since reservations relating the above refutation seemed self-evident, the subsequent publication of several of our articles was launched. They disclosed evidence of an association of the MV with further categories of cancers, as well as its absence in some distinct tumors. A mechanism by which the MV may regulate apoptosis in the tumor cells of CHL was also proposed. A suspected role for the MV in oncomodulation is advocated. We deplore, however, the absence of further interest in our hypothesis among world experts.

Keywords: classic Hodgkin lymphoma, measles virus, antigens, RT-PCR, in situ hybridization, endometrial carcinoma, breast cancer, lung cancer

1. Introduction

Classic Hodgkin lymphoma (CHL) stands out among malignant tumors, by the relative paucity of the tumor cells in the involved lymph nodes. These Hodgkin and Reed-Sternberg (H/RS) cells (**Figure 1**) are distributed unevenly within the majority of inflammatory cells, fibrosis and necrosis [1]. It is understood that, independent of the proportion of H/RS cells in CHL, the outcome of the malignancy will be favorable, with a cure rate of 75–90% [2, 3]. Such a therapeutic success is unusual for cancer at large.

When considering the many prognostics and risk factors regarding CHL, the stage of the tumor, as expected, is the most relevant. Evidence of systemic symptoms: fever higher than 38°C for at least a month; weight loss of more than 10% of body weight, lasting 6 months or more, and/or drenching night sweats, singly or collectively, worsen the prognosis [1]. A difference is described between genders, as females fare as a rule better than males, no consideration being given to a higher incidence of nodular sclerosis CHL in females. Indeed, children and older adult males with mixed cellularity CHL, especially in developing countries, get on worse with the disease [2, 4]. In contrast, females would be included in the bulk of CHL patients, aged 15–36, with a favorable outcome [5].

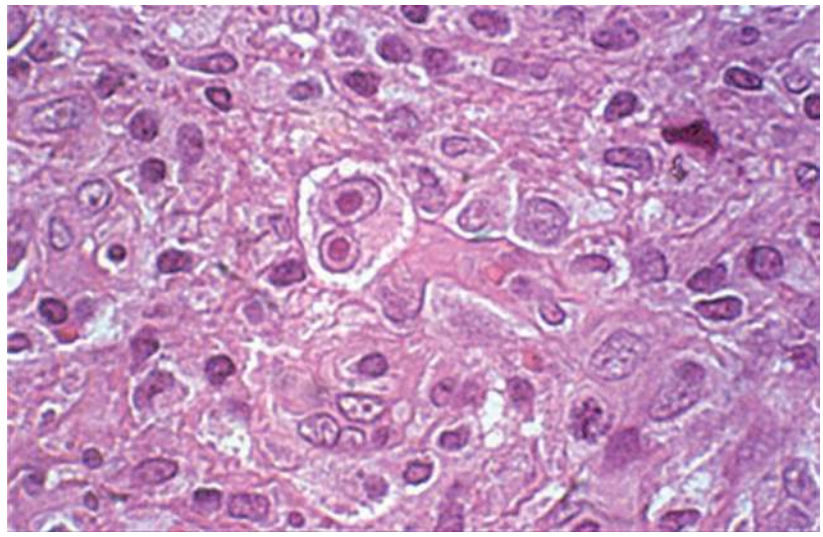

Figure 1.
Section of lymph node from a patient with classic HL. Off center is a Hodgkin/Reed-Sternberg giant tumor cell (H&E × 360).

Some 15-20% of human cancers are thought to arise by mechanisms which concern viruses, bacteria, or parasites [6, 7]. Evidence for their involvement comes partly from the detection of the microorganism in biopsies and partly from epidemiological and animal studies. Viruses might modify cellular genes directly, as with RNA tumor viruses, or may affect cell proliferation, as with DNA tumor viruses, and thus contribute to neoplasm formation [6, 7]. As a rule, the virus is responsible for only a limited number of steps in the promotion or progression of cancer. In most cases, the precise role of cancer-associated virus is hard to decipher, due to the long delay from the initial viral infection [6]. In any case, the number of human beings infected with the above viruses is much larger by far from that of patients who develop cancer. Therefore, tumor viruses probably act in conjunction with additional factors [7].

Chronic inflammation may play an important role in the above processes as well as in other pathologic events which may lead to cancer [8]. In some cases of unresolved chronic inflammation, the immune response becomes maladaptive, hence promoting tumorigenesis [8]. A regenerative process supported by an array of bioactive mediators, promote cell survival, tissue remodeling, and angiogenesis. The mediators will also cause genomic stress and mutations [9].

A correlation between CHL and the Epstein-Barr virus (EBV) has been demonstrated many years ago [10, 11], with a causal relationship being established as soon as 2001 [12]. The incidence of EBV infection in CHL has shown a wide geographic variation, from 17 to 30% in industrialized countries, to as high as 100% in some developing countries [13, 14]. Several more years elapsed before the distribution of EBV infection by gender, age, and CHL type was determined (**Figure 2**) [13, 14]. Thus, EBV is expressed more frequently in males, notably in children from developing countries, as well as in older adults all over the globe, these two groups showing as a rule mixed cellularity CHL. Female CHL patients stand out, in general, by showing nodular sclerosis CHL with a markedly lower level of EBV expression and a young adult age (15–36) [15]. A small subset of young adult males will develop CHL after recovering from infectious mononucleosis [16]. The EBV is recognized as oncogenic. Specifically, its transforming potential, with cell proliferation, inflammation, and apoptosis arrest, has been related to the nuclear factor-κB (NF-κB) and the EBV/LMP1 will further contribute to the inhibition of the H/RS cell apoptosis [15–19]. As noted, EBV does not play a significant role in the largest subset of CHL that affect young adults and most of the female CHL patients.

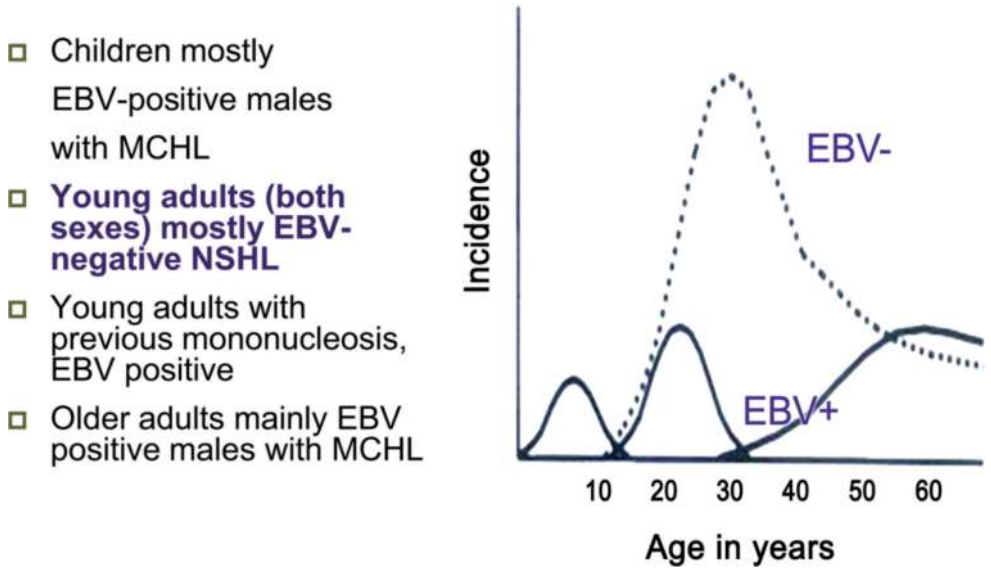

Figure 2.
Association between age, gender, EBV expression, and classic Hodgkin lymphoma, including the types thereof.

The above subset of young adult female patients corresponds to that defined in the hallmark epidemiological studies in the early 1980s as patients with the "late host response model" [15–19]. These patients are considered to be submitted to the late exposure to a common infectious agent, the term "late" meaning that instead of coming across the common infectious agent for the first time in early childhood, this occurs at adolescence or young adulthood. The epidemiological thesis further suggests that these individuals had a late encounter with the agent, since they had few and late contacts with other children (single child, individual family house, and child who skipped the kindergarten) [15]. To account for the late host response model, it is suggested that a common agent, probably a virus, reaches the organism for the first time at a stage in which the metabolism and/or the hormonal balance is markedly different from that of a toddler, the age at which the encounter regularly takes place. At this point, the internal environment has varied deeply and probably, so has the immune system [15–19]. Thus, it seems that the EBV plays a relatively minor role, in the largest subset of the CHL that is most prevalent in industrialized countries [18].

When looking for a substitute for the EBV in CHL, it was observed that none of the additional viruses, HHV-6, -7, -8, polyoma JC virus, SV40, HTLV-1 and -2, and human retrovirus 5, once considered as possible candidates, have been detected in CHL tissues [20–26]. The only virus to be incompletely excluded at that time was the HCMV. However, one distinct virus showed qualities and associations which recalled to various extents of those of CHL.

The "candidate" proposed by us is the measles virus (MV). It penetrates the organism through the oropharynx. But, since it is lymphotropic, it enters the adjacent lymphoid tissues. This may account for the vast majority of CHL starting in cervical and/or in mediastinal lymph nodes [12, 27]. Histologically, it elaborates multinucleated cells—the Warthin-Finkeldey polykaryons. Of note, these polykaryons differ largely from the H/RS cells of CHL, mainly by the number of nuclei (up to 70) as well as by their phenotype (they are T-lymphocytes) [28]. An acute measles infection is soon followed, on the one hand by a life-long specific immunity, but also, paradoxically a transient cellular immune deficiency systematically develops. This deficiency lasts about 6 months, but at times, may last longer. The cellular immune deficiency which affects CHL patients is not different in principle, but it may be long lasting [12].

At the time our thesis was formulated, the type of MV neural infection known as subacute sclerosing panencephalitis (SSPE) was considered one of the very few persistent MV infections, most being CNS slow viral infections. However, it was difficult to grasp a possible association between MV and cancer, which would not account for the long lapse of time necessary for the malignant transformation. In that context, the absence of a persistent MV infection might be counterproductive. The alternative being that against all odds, MV may be oncogenic, and the MV is not among the consensually recognized oncogenic viruses! An additional clue to MV causing a persistent infection beyond the CNS is the absence of membrane expression of hemagglutinin MV antigens, although this lack of expression might be due also to the lymph node mode of fixation [29].

2. Materials and methods

The basic part of our study (2003–2004) concerned 154 patients with CHL diagnosed between 1975 and 2000 at the Soroka University Medical Centre in Beer-Sheva, Israel, whose tissues were available for research. Only pretreatment biopsies with confirmed histological type, by two hematopathologists were included. Clinical data were retrieved from the patients' charts and when those were not found, the details were searched in the Israel Population Registry by linkage.

2.1 Immunohistochemistry

Immunophenotyping was performed on formalin-fixed, paraffin-embedded (FFPE) tissues by the avidin-biotin peroxidase complex method and the Vectastain kit by Vector Laboratories (Burlingame, CA, USA) as described. The EBV/LMP1 antibody was purchased from Dako (Glostrup, Denmark); the anti-MV antibodies were either commercial: anti-nucleoprotein (NP)-MV; anti-hemagglutinin (HA)-MV; anti-matrix (M)-MV; and anti-phosphoprotein (P)-MV were purchased from Chemicon International Inc. (Temecula, CA, USA). Further experimental antibodies were a gift from Birrer et al. [30]: anti-NP, H14; and from Schneider-Schaulies et al. [31]: anti-NP, L39/22; L39/61 or anti-HA antibodies, K83; L77. The antibody specificity had been demonstrated by Western blotting. The staining was considered positive if 10% of H/RS cells or more were positive. For stringency purposes, we requested positivity for two MV antigens, for a case to be considered positive. A murine neuroblastoma cell line permanently infected by MV was used as a positive control; the same cell line, but non-infected was used as a negative control. These cell lines were also the controls used for the RT-PCR and ISH assays. Twenty-five cases of non-Hodgkin lymphoma were also tested by IHC.

2.2 Reverse transcriptase-polymerase chain reaction

Total RNA was obtained by extraction of snap-frozen lymph node samples from part of our patients, when available. For this goal, EZ-RNA Total RNA Isolation Kit (Biological Industries Co., Beit Ha-Emek, Israel) was employed, using the manufacturer's instructions. To each sample, 40 U of RNA-guard (Promega, Madison, WI, USA) were added. One step RT-PCR was performed with Ready To Go ™ RT-PCR beads (Amersham Pharmacia Biotech Europe GbmH, Freiburg, Germany). A measure of 3 μl of RNA (2-6 μg) was used for each reaction. For the primers used in this study, see Table 1 from Ref. [3]. Nested PCR was carried out, using SUPER-NOVA DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany).

Total RNA was also retrieved from several FFPE tissues from CHL patients, using the Paraffin Block RNA Isolation Kit (Ambion Inc., The RNA Company, Austin, TX. USA) and RT-PCR followed by nested PCR carried out with 10 μl RNA, as described above. As controls, we used seven cases of non-Hodgkin lymphomas, one CHL which was negative for MV by IHC. As housekeeping genes, we used β-actin and protein phosphatase 2C β (pp2Cβ) considered appropriate for experiments related with low amounts of RNA.

To confirm our RT-PCR findings, the cDNA products of the nested PCR were blotted to GeneScreen (NEN Research Products, Boston, MA, USA) and hybridized to either an NP-MV probe (*CAATCCCTGGAGATTCCTCA)* or to a HA-MV probe (*GCTGGAAGCTGACACCTTTC)*, labeled [γ-^{32} P] ATP (Amersham).

2.3 *In situ* hybridization on paraffin sections

The digoxigenin (DIG)-labeled antisense and sense probes to MV-specific mRNAs were prepared by linearizing the plasmids containing NP-MV including positions 105–586, and those including HA-MV encompassing positions 8147–8482 in PGEM-T Easy Vectors (Promega), with *Sal*I or *Sac*II. *In vitro* transcription was performed with the DIG-RNA Labeling kit, with SP6 for NP antisense and HA sense probes and T7 for NP sense and HA antisense DIG-labeled probes. The quality of the probes was determined by dot blotting, using a DIG-DNA detection kit (Roche Molecular Biochemicals), according to the manufacturer instructions, with minor modifications. *In situ* hybridization was performed, using Ogata's technique with certain modifications [32]. Paraffin sections were deparaffinized, rehydrated, and submitted to microwave in the presence of 10 mM MgCl2 buffer (pH 6) for 5 min at 750 W. Sections were allowed to cool down for 20 min and then digested with 20 μg/ml proteinase K for 10 min at 37°C. They were then fixed in paraformaldehyde in 0.1 M phosphate buffer containing 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/ml tRNA, 1 x Denhardt's solution, 10% dextran sulphate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA, pH 8.0. Hybridized DIG-labeled probes were detected by the DIG-Nucleic Acid Detection kit (Roche Molecular Biochemicals), followed by blocking with 1.5% of the blocking reagent included in the kit. The color reaction was stopped with 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA. Sections were then fixed in 4% paraformaldehyde in phosphate-buffered saline and stained with hematoxylin. In addition to the neuroblastoma cell lines used as positive and negative controls, CHL cases negative for MV by IHC and NHL served as controls.

2.4 Comparison between IHC, RT-PCR, and ISH

This was performed on a sampling of cases. For this purpose, IHC positivity to a single NP-MV or HA-MV antibody was considered sufficient.

2.5 Clinicopathological correlations

Emphasis was given to MV IHC and to EBV-LMP1 expression, as RT-PCR and ISH disclosed much less results. For contingency table analysis, the $\chi 2$ or Fisher's exact test was used as appropriate.

3. Preliminary results

The lymph node tissues of 82 of 154 CHL patients were positive for at least two MV antigens (**Figure 3**). Neutralization of immunostaining was made possible by

incubation of the primary anti-NP-MV antibody with the corresponding NP-MV peptide (**Figure 3b**). This assay confirms the specificity of the anti-NP-MV antibody in this context. Twenty-five cases of non-Hodgkin lymphoma (NHL) were totally negative for MV antigens.

A Southern blotting of the nested PCR shows several bands, some stronger, others faint, disclosing more hemagglutinin (HA, H) MV positivity, as compared with NP-MV positivity (**Figure 4**). Samples from 7 NHL were negative. Thus, in our laboratory, HA-MV RNA was positive in 4 of 15 cases, while only 2 of 16 NP-MV RNAs were expressed by the Southern blot in CHL.

Upon sequencing and alignment of three of the experiment products, we displayed an almost complete homology with the control (see [3] for details on the homology).

In situ hybridization on paraffin sections shows a strong cytoplasmic staining in the positive control (persistently MV infected murine neuroblastoma cell line) (upper left). The same cell line uninfected by MV was negative. An H/RS cell shows strong cytoplasmic positivity with NP-MV (lower left). Positive granular staining of several H/RS cells is disclosed in the right lower quadrant (**Figure 5**). Altogether, 2 of 7 CHL sections submitted for ISH were positive for HA-MV RNAs, while 8 of 21 were positive for NP-MV RNAs.

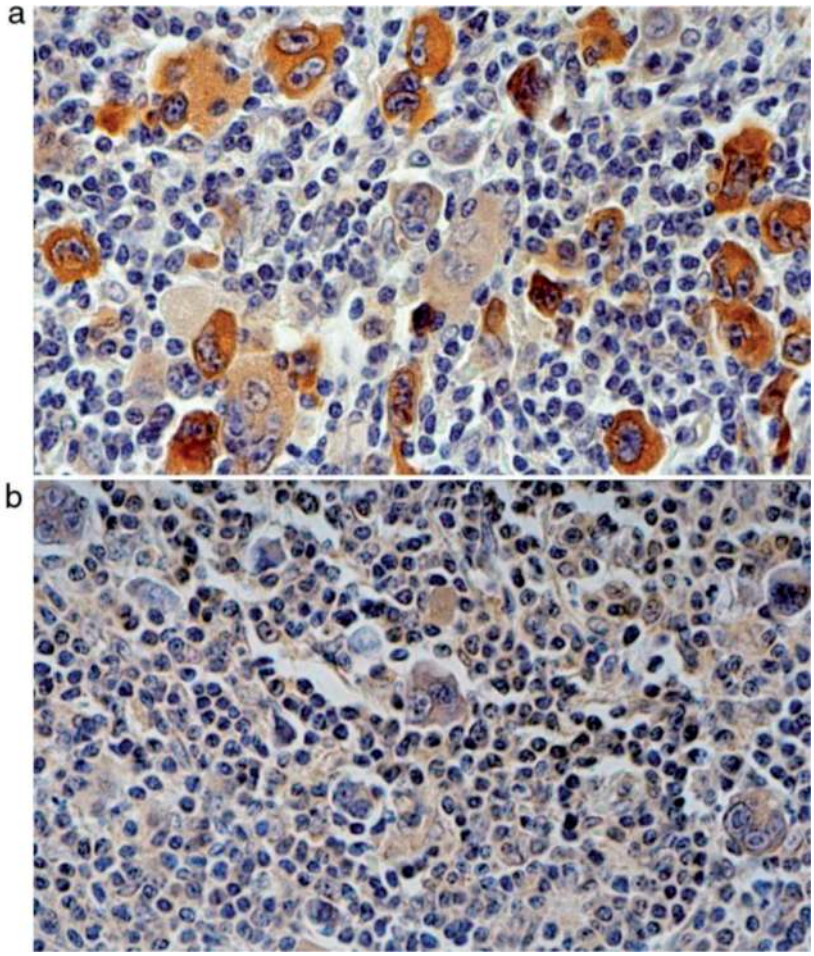

Figure 3.
Classic Hodgkin lymphoma-nucleoprotein-MV expression. a. Positive cytoplasmic immunostain of Hodgkin/Reed-Sternberg cells. b. Immunostaining following neutralization of antibody with a relevant MV peptide (Immunoperoxidase with DAB × 320).

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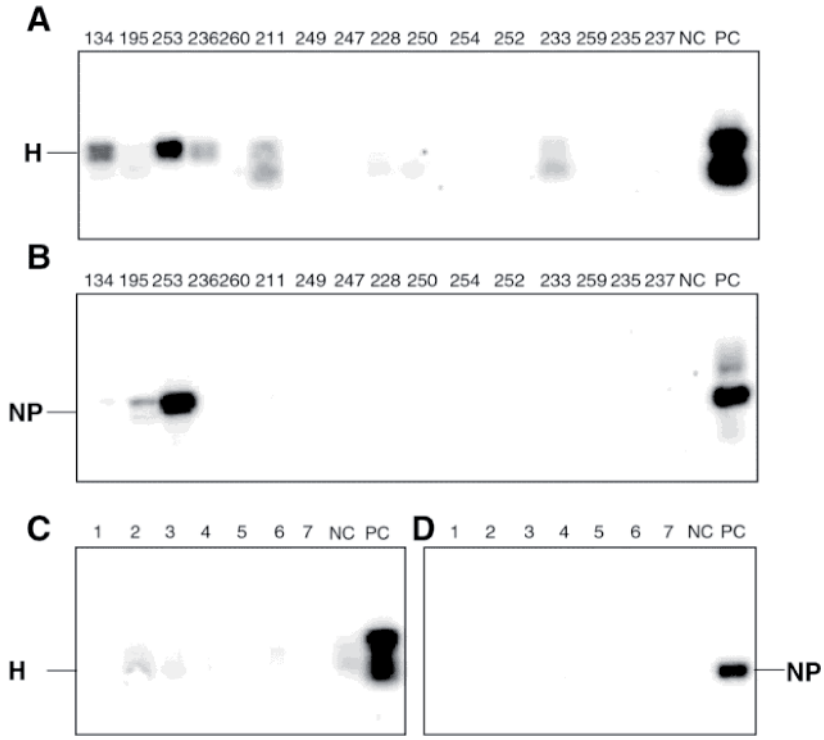

Figure 4.
Southern blotting based on cDNA from RT-PCR: A. A few cases of CHL show bands positive for hemagglutinin-MV (PC—positive control). B. Isolated bands, positive for nucleoprotein-MV. C and D. Absence of expression of MV in non-Hodgkin lymphomas.

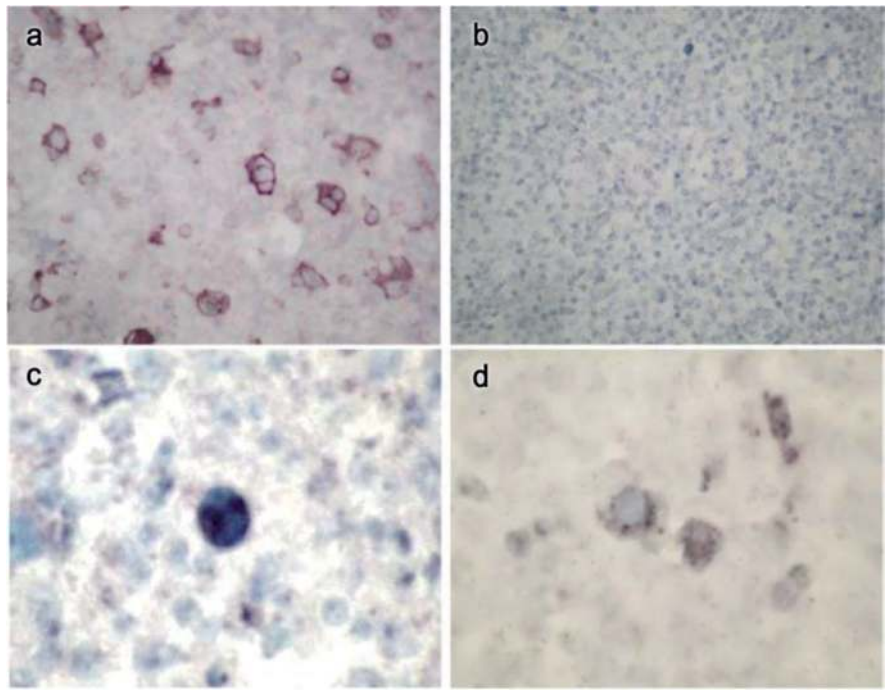

Figure 5.
In situ hybridization with MV-RNA. a. Positive control, using a murine, MV-transfected cell line. b. Negative control, with a non-transfected cell line. c. A Hodgkin/Reed-Sternberg cell disclosing positive cytoplasm for MV-NP-RNA. d. H/RS cells showing a granular cytoplasmic positivity for MV-HA-RNA.

A clinicopathological correlation was based on the immunophenotypic findings, as the specificity of the antibodies had been sustained by Western blotting and since, in each case, the immunostain had been confirmed with more than two antibodies. Last, the immunohistochemical (IHC) study had released the most manageable results.

Table 1 shows a statistically significant expression of MV antigens in female patients, in nodular sclerosis (NS) CHL, as compared with mixed cellularity (MC) CHL. A significant correlation was exhibited between negative CD15 and p53, and the negative expression of MV. **Table 2** displays a statistically significant expression of MV antigens compounded by the expression of EBV/LMP1, in NS-CHL, significantly more than that in MC-CHL, in early clinical stages, with apoptotic index lower than median and with the positive expression of MDM2.

A multivariable logistic regression model of the association of prognostic factors with dying from CHL, in 89 patients, is shown. Stage IV, as compared with the remainder, carried a significantly poorer prognosis, thus confirming that our cohort was representative. The compound expression of MV and EBV, specifically of MV+; EBV−, as well as MV−; EBV− was displayed as poor risk factors in our patients (**Table 3**).

		MV+	**MV−**	***p* Value**
		n (%)	n (%)	
Total		82 (54.3)	69 (45.7)	
Gender	F	40 (**64.5**)	28 (35.5)	0.036
	M	42 (47.2)	47 (52.8)	
Subtype	NS	58 (**68.2**)	27 (31.8)	0.0013
	MC	21 (40.4)	31 (59.6)	
CD15	pos	77 (59.7)	52 (40.3)	0.015
	neg	4 (26.7)	11 (**73.3**)	
p53	pos	72 (61.5)	45 (38.5)	0.017
	neg	5 (31.3)	11 (**68.8**)	

Table 1.
Associations of classic Hodgkin lymphoma with the measles virus.

		MV+; LMP−	**MV−; LMP+**	***p* Value**
Subtype	NS	45 (**86.5**)	7 (13.5)	0.00008
	MC	11 (44)	14 (56)	
Stage	I–IIA	22 (**84.6**)	4 (15.4)	0.023
	IIB–IVB	22 (57.9)	16 (42.1)	
BCL-2	Pos.	31 (**93.9**)	2 (6.1)	0.0001
	Neg.	27 (54)	23 (46)	
MDM-2	Pos.	36 (**87.8**)	5 (12.2)	0.0013
	Neg.	18 (54.5)	15 (45.5)	
Apop index	<Median	31 (**81.6**)	7 (18.4)	0.024
	>Median	22 (42.1)	16 (57.9)	

Table 2.
Hodgkin lymphoma—association with measles and Epstein-Barr viruses.

Factor	Odds ratio	95% CI	*p* Value
Gender	1.32	0.34–5.18	0.69
Stage	**13.67**	2.3–80.5	0.004
Bcl-2	0.34	0.08–1.44	0.34
MV+; EBV−	**10.05**	0.98–103.47	0.05
MV−; EBV+	1.53	0.07–35.44	0.79
MV−; EBV−	**15.2**	1.05–220.3	0.04

Table 3.
Multivariable logistic regression model of association of prognostic factors with dying of HL in 89 patients.

4. Interim discussion

The results of the first part of our study (2004) are summarized [3]. The association between MV and CHL, in a descriptive investigation, does not contain, so far, causal elements. This linkage is sustained by older epidemiologic studies describing brain and spinal cord tumors, as well as CHL, which followed exposure to the MV around the time of birth [33, 34]. They also suggest sporadic cases of CHL regression, occurring after acute measles infection or an MV vaccine [35–38]. Moreover, viral childhood infections, including measles, have been reported to protect from CHL occurrence [39].

Early clinical correlations exhibit more females, more NS-CHL, and more early stage cases, among patients with positive MV expression in their lymph node tissues. Nevertheless, positive MV expression in CHL seems to be related with a worse prognosis. One cannot refute the loss of the advantage conferred to the above variables by an MV infection, as expressed in the H/RS cells of CHL. Any mechanism involving the MV in CHL, if uncovered, should correlate with a worse outcome of the malignancy.

5. The refutation episode

Two years following the publication of the above-mentioned article and several months after the presentation of a poster at the Hodgkin Lymphoma Meeting in Kiel, a scientist, from Germany, contacted me indirectly, informing me that he wanted to investigate our findings. He proposed to find out whether he could reproduce our data in his lab. He also requested to make any fresh-frozen CHL tissues available to him, mainly those which showed an MV-antigen positivity.

In their labs, the German scientists had collected 44 frozen CHL samples, of which, only 18 were selected for their high quality RNA, as determined by the Agilent technology, as well as for classic H/RS cell morphology. Using UV-laser beam single cell microdissection, some 100 H/RS cells were pooled for each experiment. Total RNA was extracted and RT-PCR was performed with primers from three MV genes, which were different from those we had used in Israel [40].

Five CHL-derived cell lines were found to be negative for the three MV transcripts. Moreover, the 18 selected German cases did not show, either, evidence of any of the three viral transcripts. The GAPDH gene, used as a housekeeper gene for these experiments, displayed a large amount of RNA. At this point, the German group had received the 22 snap-frozen CHL samples from our laboratory. The cases had been chosen, as requested for their positive MV antigens, as shown on FFPE tissues. However, the selection highlighted only 7 of these cases, chosen for the high RNA quality and the classic H/RS cell features [40].

	NP-MV		HA-MV	
	Positive/n	**(%)**	**Positive/n**	**(%)**
RT-PCR	1/16	(6.25)	7/21	(33.3)
ISH	7/14	(50)	4/7	(57.1)
IHC	25/29	(86.2)	20/29	(68.9)

NP, nucleoprotein; HA, hemagglutinin; MV, measles virus; RT-PCR, reverse transcriptase-polymerase chain reaction; ISH, in situ hybridization; and IHC, immunohistochemistry.

Table 4.
Comparison of results obtained by three assays.

All 25 RNA samples disclosed negative results with the three MV transcripts, as studied in Essen labs. Back to Israel, 5 of 12 German histological unstained slides, subjected to IHC, were found to express MV antigens in the H/RS cells. A similar discrepancy in favor of IHC was also obtained in our series (**Table 4**).

The German scientists invited one of my colleagues and myself to co-author their manuscript. At every stage, we were reluctant to accept the invitation, due to the concern for a permanent damage to our hypothesis. Moreover, we did not curb our criticism, which covered several aspects of the manuscript. Prominent among these was the tight selection of the cases (both those from Germany and Israel). The methods used in Essen differed markedly from ours, notably the single cell micro-dissection, a different strategy for the RT-PCR and the choice of primers. Following our criticism, minor changes were introduced, but the German group did not go all the way: "….due to their worry that the manuscript will lose its sharp edge and might not be accepted by a highly rated journal."

The Scott collaborators of the Essen scientists reported above selected a group of patients, many of whom having suffered from measles, mainly as children. We consider this selection as counterproductive, since these patients should have been protected from the development of CHL. The very fact that they were not refutes the prevalent thesis that exposure of a child, aged 1–4 years, to MV infection protects the subject from CHL at a young-adult age [41]. The Scottish group may have had their own agenda, which might have unsettled the question raised by us. In addition, the antibody used by the Scott group is problematic: the clone 49–41 was purchased by us from Argene-Biosoft, Varilhes, France (# 11-045). It was not an anti-nucleoprotein antibody, as stated, and was positive in our lab for 37 of our 72 CHL cases (data not shown). Moreover, not unlike the German scientists, the Scotts used the GAPDH gene as a housekeeping gene, and we have already mentioned why it was not appropriate.

I have to admit, that we not only considered ourselves as the minor partner, but that we were such indeed! We had been recurrently reassured by the German scientists and by then had our two names included in the manuscript as co-authors. However, when we read the finalized doublet articles (in addition, the article by Wilson et al., weighted heavily on the balance) [41], published in the International Journal of Cancer, we realized that we had made a huge mistake.

The days which followed the double publication were crucial, since we were not sure, as co-authors, whether we were entitled to carry out an "insider" censure. When we read the final paper, we grasped the experience in terms of a possible deception. In addition to the impediments mentioned above, we also noted that the housekeeping gene, the GAPDH, might not be an adequate choice at all, since we had shown that CHL is most probably of low MV-RNA abundance. In contrast, the Essen group had described in relation with GAPDH a large amount of RNA [40]. Moreover, of the seven Israeli cases, selected for the experiment in Germany, five had been shown by us to present a faint MV-RNA positivity only (two) or were negative (three).

An independent putative evidence for a role of the MV in carcinogenesis is that part of the MV proteins that interacts with and stabilizes the Pirh2 protein, an ubiquitin E3 ligase, by preventing its ubiquitination. Pirh2 indeed negatively regulates p53 by targeting it for degradation in the proteasome.

Altogether, our criticism of the article, in which we took officially a part, is in principle that the German scientists carried out an investigation, which was by their declared intention, a duplication of our own research [3]. However, their study was anything, except a duplicate of ours! It stands out independently, with major flaws, notably a very strong selection of cases, the use of questionable primers of a housekeeping gene which is not appropriate for the expected amount of retrieved MV-RNA. It is of note that when confronted with our criticism regarding the manuscript, the German group seemed to agree, but did not modify the manuscript: "…for fear of weakening our data". Moreover, they insisted on mentioning the relevance of the findings to the CHL pathogenesis, which was not appropriate at this stage nor was it agreed upon [40]!

At this point, we insist that the impact of the European group refutation, on a possible role of MV in CHL, was extremely ruinous to our position and thesis. Moreover, its repercussions are felt to this day: for the reasons mentioned above, not a single investigator has accepted to engage in this project anymore, together with us or independently. By so doing, the two research groups have categorically "buried" our hypothesis. At the same time, we felt a release from the constraints which might have led us freeze in our position and we allowed ourselves again to indulge in the research concerning the measles virus and cancer. Therefore, we first wrote a review on our experience, as described above [42, 43].

6. New evidence

We set out to delve into endometrial carcinoma (EC), looking for the footprints of the MV in this cancer. Our cohort of EC included 49 patients, 36 of whom had a known follow-up. Using IHC and several anti-MV antibodies, we disclosed that 26 of the 36 (72%) did express MV antigens in tumor cells, mainly in type I EC (**Figure 6**). In addition, 16/21 of the patients displayed positivity for MV-RNA by ISH. When type II EC was allied with MV, then deeper myometrial invasion and a higher mortality were evident [44].

Our next endeavor concerned the possible role of MV in lung cancer. Our interest was first raised by the probable relevance of Pirh2 in lung cancer. Indeed, Pirh2, an E3 ubiquitin ligase, was found to be expressed in lung cancer, in which it inhibits p53 [45]. It was later found that the P-protein of the MV inhibited the function of Pirh2 (see description in [48]). This indirect association between lung cancer, Pirh2 and p53, and MV led us to the following study. We identified 65 non-small cell lung cancers, for which FFPE tissues were available over 10 years. We limited our study to the IHC analysis with several anti-MV antibodies (**Figure 7**). Of the cases included, 54/65 were positive for MV antigens. These 54 positive cases were associated with older patient age, with positive Pirh2 expression, as well as with an improved survival [46].

The following study concerned breast cancer and the MV-IHC expression. We identified 131 breast cancer cases, with preserved FFPE tissues. Of these cases, 64% were positive for the MV antigens (**Figure 8**). Positive MV expression was associated with ER ($p = 0.018$); with Ki-67 < 40% ($p = 0.029$); with low or intermediate grade ($p = 0.037$); with age < 50 ($p = 0.039$); and with p53 overexpression ($p = 0.049$) (**Table 5**). It seems, therefore, that the MV may play a role in the development of this cancer [47].

We have speculated whether the MV phosphoprotein, through its relation with the Pirh2, ubiquitin ligase, by itself negatively related with the p53 expression, may have more relevance, *a priori,* than other MV antigens to the pathogenesis of the

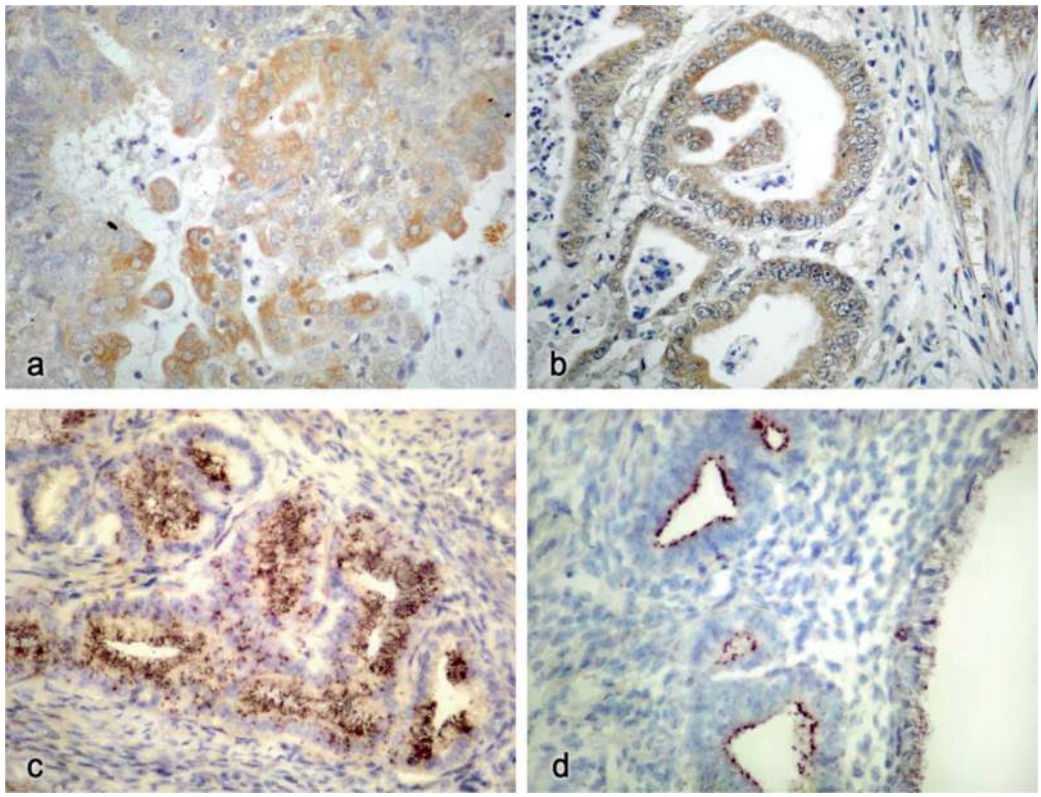

Figure 6.
Endometrial carcinoma—MV expression in tumor cells and in normal endometrium. a. Cytoplasmic HA-MV antigen is positive. b. Positive immunostain for NP-MV antigen. c. ISH positivity for NP-MV in tumoral glands. d. ISH positivity of surface and glandular normal epithelium of the case shown in c.

MV-associated cancer [48]. We therefore purchased a robust anti-MV-phosphoprotein antibody (9H4): sc-101,356, Santa Cruz Biotechnology, and used it by Western blot, first, to determine whether we could find a variance between the different types of lung cancers and in addition to determine whether the normal lung tissues around the tumor did express the MV. But, the distribution of lung cancer types in our cohort was clearly in favor of adenocarcinoma, which expressed the P-MV in 11 patients (84.6%). This was also the rate of its expression in the normal lung tissue

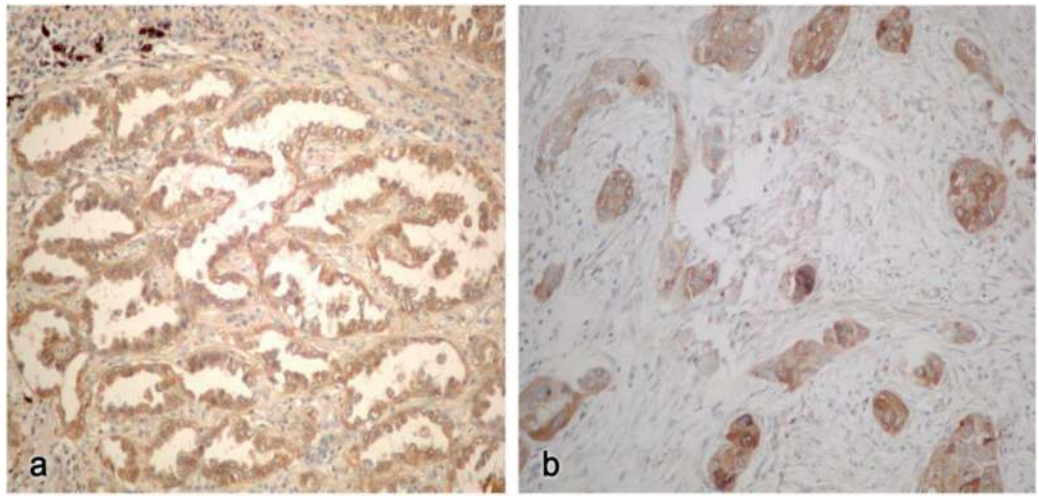

Figure 7.
Lung cancer—positive for MV. a. Adenocarcinoma. b. Squamous cell carcinoma (IHC with DAB × 240).

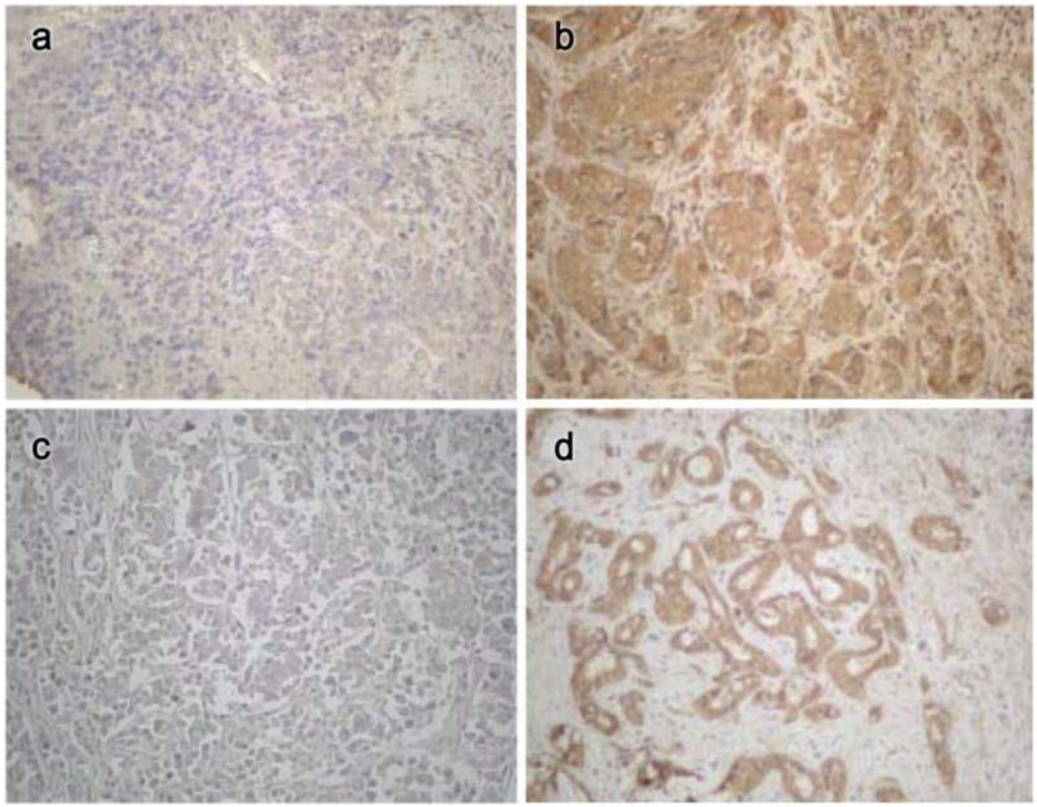

Figure 8.
Breast cancer is positive for measles virus antigens. a and c. Negative controls. b and d. Positive immunostains (immunoperoxidase with DAB × 180).

Variable	Variable distribution	
Demographics		
Age	<50 years	>50 years
Ethnicity	Jewish	Bedouin-Arab
Birth place	Israel/Europe/America	Asia/Africa
Pathology		
Tumor diameter	<10 mm	>10 mm
Axillary LNs	None	Involvement
DCIS component	Yes	No
Grade	Low/intermediate G1–2	High G3
Immunohistochemistry		
ER	Positive	Negative
PR	Positive	Negative
HER2	Positive	Negative
Ki67	Positive	Negative
p53	Positive	Negative
NP-MV	Positive	Negative
HA-MV	Positive	Negative

Table 5.
Distribution of demographics, clinics, pathology, and MV expression in breast cancer patients.

Pathology	Positive (%)	Negative (%)
Adenocarcinoma	11 (84.6%)	2 (15.4%)
Squamous cell carcinoma	3 (100%)	0
Large cell carcinoma	1 (100%)	0
Bronchioloalveolar carcinoma	1 (100%)	0

Table 6.
Western blotting using a phosphoprotein-MV antibody.

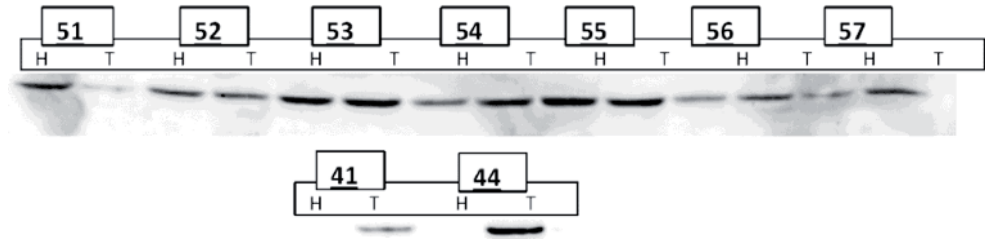

Figure 9.
Western blotting with an antiphosphoprotein-MV antibody on fresh frozen tissues of non-small cell lung cancer. Most cases show positive expression both in tumor tissue (T) and in nonadjacent nonneoplastic lung tissue (H). In a few cases, the MV expression is found in the tumor only.

	Positive staining %			Negative staining %		
Organ	**Nucleus**	**Cytoplasm**	**Stroma**	**Nucleus**	**Cytoplasm**	**Stroma**
Lung	10 (52.6%)	2 (11.1%)	0	9 (47.4%)	16 (88.9%)	18 (100%)
Colon	13 (68.4%)	7 (36.8%)	4 (23.5%)	6 (31.6%)	12 (63.2%)	13 (76.5%)
Stomach	14 (73.7%)	4 (21%)	0	5 (26.3%)	15 (79%)	19 (100%)
Breast	15 (75%)	10 (50%)	2 (10%)	5 (25%)	10 (50%)	18 (90%)

Table 7.
Tissue microarray using a phosphoprotein-MV antibody.

(**Table 6**; **Figure 9**). The second step was to look for the expression of the P-MV in a tissue microarray (one tissue microarray slide). We compared 10 lung cancers, 13 colon cancers, 14 stomach cancers, and 15 breast cancers. Breast cancer expressed the MV antigen in 75% in the nucleus and 50% in the cytoplasm. Stomach cancers expressed the antigen in 73.7% in the nucleus and 21% in the cytoplasm. Colon cancers expressed the MV in 68.4% in the nucleus (**Table 7**). The tissue microarray study had demonstrated a nuclear expression of MV antigens, of which we have not been aware before. This may be due on the one hand to the tiny size of the tissue section. On the other hand, it may be specific to the present anti-P-MV antibody. In any case, various immunostains have shown a marked shuttling of antigens between cytoplasm and nucleus in various tissue sections and with varied antigens [49] (**Table 7**).

Our experience with melanoma and benign melanocytic nevi is shown in **Table 8**. The table shows the expression of several MV antigens and of Pirh2. The expression of NP-MV is the strongest and resembles that of Pirh2. A similar trend, but with a more limited expression, is found in benign melanocytic nevi. It is of note that the expression of the P-protein in melanomas is the mirror image of that of the NP-MV antigen. This was a preliminary exercise and no follow-up is yet

Antigen	Level	Melanoma	Benign nevi	P value
NP-MV	Pos.	46	23	
	Neg.	10	1	0.16
P-protein	Pos.	19	10	
	Neg.	36	12	0.37
HA-MV	Pos.	19	7	
	Neg.	3	1	1.00
Pirh-2	Pos.	44	19	
	Neg.	12	6	0.79

Table 8.
Melanoma and measles virus.

available. The positivity of 23 benign nevi for NP-MV should be investigated to exclude evidence of a pre-malignant condition.

At this stage, we were motivated to determine whether some categories of tumors might be uniformly negative for MV antigens. One limitation is that these tumors were often showed with a restricted sampling. In our original study, we included 25 NHLs by IHC and 7 cases of non-Hodgkin lymphoma by RT-PCR. They were all negative for MV. Only later did we find an interest in ALK-positive anaplastic large cell lymphomas in this regard. In contrast, these lymphomas were predominantly positive for MV. A large scale study of seminomas showed negativity for the MV antigens. Glioblastomas and mesotheliomas were negative for the antigens (Samuel Ariad, personal communication). I later examined small samples of the following tumors which were also negative for the MV antigen: Merkel-cell tumors, hepatocellular carcinoma, malignant mixed mesodermal tumors, and desmoid tumor.

In 2010, we revised and expanded a prior study on the role of apoptosis in CHL [50, 51]. This was undertaken in the light of a consensual point of view: it has been stated that apoptosis in CHL is arrested; this is carried out not only with the help of the NF-κB machinery but also through the expression of EBV-LMP1 that inhibits apoptosis [52–55]. The consensus further determines that this apoptosis hindrance is necessary, since tumor cells in CHL are B cells which cannot express the B-cell receptor (in physiologic conditions, such cells have a strong tendency to die in apoptosis). Thus, apoptosis inhibition is a pre-condition for the survival of H/RS cells [56]. However, in our revision, we found inhibition of H/RS cells apoptosis in 55% of the 217 cases only. Our findings also suggested that NF-κB, specifically the p65 protein, as well as EBV/LMP1 expression did not correlate with apoptosis arrest, in contrast with the consensus view [57]. Of note, it has been shown that although the presence of canonical NF-κB has been considered to be implied, it is the noncanonical NF-κB, which is of predominant relevance in CHL [51]. In the revision we have shown that the most significant association of H/RS cell apoptosis was with p53, the negative expression of which was related with a high apoptotic index ($p = 0.001$). We looked for a link between positive MV expression and factors related with apoptosis. Associations with apoptotic index less than median ($p = 0.005$), with MDM2-positive ($p = 0.028$) and with IκB-positive ($p = 0.0001$), were displayed. Viruses have a propensity to regulate the apoptotic mechanisms of the infected cell. We have hereby proposed a pathogenic mechanism, involving the MV and that is effective in the modulation of apoptosis, at least in CHL [51].

7. The atypical measles syndrome

In contrast with the classic measles infection, the atypical measles syndrome occurs in older patients, often presenting with a heterogeneous rash, pulmonary infiltrates, hepatic dysfunction, and eosinophilia. The formation of immune complexes has been proposed as responsible for the manifestations of the syndrome [58]. This self-limiting disease, may show, however, long lasting, asymptomatic pulmonary nodules. An immune waning is suspected to occur in this disorder, though such an event has been refuted with measles virus infection at large. Officially, patients with this syndrome have been reported as recently as 2015 [59].

The atypical measles syndrome (AMS) stands out when compared with classic measles for the following reasons.

AMS presents at an older age, often with a high and prolonged fever. A polymorphic rash starts in the hands and feet. In the lungs, this disorder shows nodular infiltrates together with swollen hilar lymph nodes. These changes may regress suddenly. Liver function tests are often abnormal in AMS. Eosinophilia is frequent. This disease entity was first described in 1963, following the vaccination with the formalin inactivated measles virus (FIMV) vaccine. A subsequent exposure to the wild type MV has often been identified. The FIMV vaccine is said to have been withdrawn in 1967, but new cases of AMS were still reported in 2015 [59].

Clinically, the AMS does not resemble the classic MV disease and is usually mild. GPs have difficulties in making the diagnosis in real time. Anti-MV antibody titers are higher than 1:160, which is very unusual for classic measles. In AMS, Koplik spots are frequent and mostly will last 5–7 days, while they last rarely more than 2 days in the classic disease. Photophobia is rarely present in AMS. AMS is probably not contagious [60].

While most cases of AMS in the 1960s were associated with FIMV, the more recent occurrences have been associated with the live attenuated measles vaccine (LAV). Vaccination with FIMV had offered protection for a few months, after which the titers dropped suddenly [61]. We have suggested that this drop of antibody titers is most consistent with a humoral immunity waning. Cases of AMS with prior LAV vaccine have been reported, which are consistent with secondary vaccine failure. Such cases have been suggested to follow a prolonged exposure to an acute measles patient [62].

To confirm the ease by which the diagnosis of AMS is missed, 291 recruits were diagnosed with measles in 1987. They were young adults, with abdominal pain, diarrhea, and vomiting (33%); Koplik spots lasting a mean 7 days; and abnormal liver function tests (86%). The diagnosis of AMS was missed. Retrospectively, this was most certainly the diagnosis, although no anti-MV titers have been reported [63].

In 2015, Levine et al. reported on the status of the measles immunity, 20 years after the two-dose MMR vaccine was introduced. Of the cohort of recruits in 1996, 95.6% had shown positive anti-MV antibodies. In 2007, this positive data had dropped to 85%. Although the authors did not dare to make the statement, this reflected a state of waning immunity, to our opinion [64].

To summarize this issue, we have showed that the AMS differs from typical measles by various features. This is probably a disorder of immunologic nature, involving immune complexes. The AMS is presently rare, but to our opinion, this may be due to a notable under-diagnosis. The association between the AMS pathogenesis and immune waning should be confirmed. It is not excluded that this disorder be related with carcinogenesis as well [65, 66], as it has been proposed that a persistent viral infection is required for virus-associated carcinogenesis [67] and that a waning immunity might lead to a persistent MV infection.

8. Conclusions and prospects

A relationship between MV expression and CHL was provisionally established on IHC [positive with two antigens or more in 82 of 154 CHL patients]; on RT-PCR [positive in 4 of 15 cases for HA-MV RNA; for 2 of 16 NP-MV RNA], and on *in situ* hybridization [for 2 of 7 CHL with HA-MV RNA; in 8 of 21 for NP-MV RNA], as well as with several other malignancies. When focusing on CHL, this association may even be causally linked: apoptosis regulation by the MV may be one of the supportive mechanisms. Our findings are probably sustained by an increasing CHL morbidity related with a progressing occurrence of measles in young adults, in Israel [68], in Quebec [69], and in Connecticut [70], in which frequent outbursts of measles are described.

A clinicopathological correlation, based on the IHC results, was chosen, mainly due to the frequent results as compared with RT-PCR and *in situ* hybridization. In this part of our study, we confirmed an association between CHL and the MV. More females, NS-CHL cases, and more patients in the early stages of their disease were linked to the expression of MV in their tissues. In spite of these variables being, as a rule, correlated with a better prognosis, when they are associated with a positive measles expression, the net effect is a poor prognosis [3].

Regarding the refutation of our findings and conclusions by the German group, this might have been an occasion for further learning. However, the repercussions thereof, on our thesis and our findings, were such that no future was available for us in this domain. As a colleague described it, our position and our theory had been buried. Moreover, I never understood why it was done in that most unpleasant way.

A chapter on the atypical measles syndrome was introduced at the end of the study. This disorder is related with measles and with the MV, but the link has not been clarified completely. Most of the AMS features, clinical, radiological, and biological, differ completely from those of measles. While measles is predominantly an infectious disease, and is highly contagious, AMS is probably not contagious, with its pathogenesis probably based on the formation of immune complexes. One of the highlights of the AMS is an anti-measles antibody titer of 1:160 or higher, much beyond that found in measles. Our interest, at this point, laid in evidence pointing toward immunity waning, as an essential component of AMS. Moreover, in the presence of a sharp drop in the titer, following mainly the FIMV vaccination, as well as occasionally that of the LAV vaccine, the subject might be submitted to the protracted exposition to an acute measles patient. So far, we are not aware of cancer developing in the context of AMS cases, but, since this disorder is often missed, it might be worthwhile looking for cases of cancer within such a context [60].

The prospects and suggestions for proceeding with our research include an epidemiologic study as well as the duplication of our basic investigation, but this once by scientists of good faith. The epidemiologic investigation should have 3 components: the first two, based on our CHL cohort, about half of which show IHC evidence of the MV. Of this population, we will attempt to identify, via the Israeli Ministry of Health Registry, patients who had contracted measles as well as those who had been vaccinated for MV, including vaccination details. The third will deal with AMS, based on the cohorts highlighted by Giladi M et al. [63], and Levine H et al. [64]. Confirmation in the first of the diagnosis of AMS, and research of patients with possibly MV-related tumors and in the second, confirmation of the relevance to immune waning.

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Conflict of interest

The author declares no conflict of interest.

Notes/thanks/other declarations

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