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Is There an Infectious Agent Behind Prostate Cancer?

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1. Introduction

This CHAPTER deals with a more defined and specified issue: whether we can already identify, point our fingers toward a specific infectious agent or infectivity pathway most likely targeting and lurking behind prostate cancer (PCa). This issue became quite evident in the past 5-6 years, in view of the heated debate on the possible role of a what was considered a novel retrovirus: Xenotropic Murine Related Virus, or XMRV, in the aetiology of PCa and subsequently also of Chronic Fatigue Syndrome (CFS). Over two years ago, the same issue was discussed by the author at the International Congress on Muscle Fatigue held in Pisa in July 2010. That presentation has been now transformed in a paper, which is in press in the journal *Neuro Muscular Disease* (NMD, Springer Verlag) [1]. The reader is therefore referred to that article -most likely already published by the time of this book printing- for aetiological considerations on CFS [1]. In this section, I will more extensively discuss the association of XMRV with PCa. Such an association was the first one to be discovered and this finding was the basis for also searching XMRV in CFS. In CFS, the potential association with an infective agent doesn't appear to be trivial, since "fatigue" has been widely associated with several types of cancer in the so called Cancer Related Fatigue (CRF), also discussed more extensively in the NMD paper [1] [2].

2. Discovery and falsification of XMRV

2.1. Linkage RNASEL – HPC-1

XMRV isolation was not a sudden or isolated finding, but it rather stemmed out of approximately twenty years of research by several groups, with a leading role by the group of R. Silverman [3] [4]. This work, as well, has even older roots, since it was initiated by decipher-

ing the antiviral response triggered by Interferon (IFN). Robert Silverman's work was pioneering and seminal in this effort: together with Ian Kerr, he clarified the Interferon (IFN) response to viral infection, initially by characterizing the 5'-triphosphorylated, 2',5'-linked oligoadenylates or 2-5A, a second messenger in the IFN response and its synthesizing enzyme (oligo-2',5'-A synthetase, or OAS) and finally discovering that 2-5A is the activator of an endogenous RNase activity, called RNase L [5] [3]. This is ubiquitously distributed but inactive inside cells, but it becomes strongly activated by binding 2-5A. By using radiolabelled 2-5A as probe, Silverman was able to identify and clone the gene *RNASEL* and to map later its location on chromosome 1q25 [5]. After approximately ten years, these studies intersected a totally different discovery path. Linkage studies on families with increased hereditary risk of prostate cancer, identified in 2002 the prostate carcinoma susceptibility gene (*Hereditary Prostate Carcinoma 1, HPC-1*) on chromosome 1q25, the same of *RNASEL* location [6]. Different alleles on this locus were associated with higher risk of PCa, such as the R462Q variant, which appeared to provide a 50% risk increase, while homozygosity doubled the risk [7]. This association between a locus behaving as a Tumor Suppressor Gene (TSG) and an Anti-Viral Response (AVR) gene is strongly suggestive of viral involvement in PCa. In the July 2010 presentation at the International Meeting on Muscle Fatigue -which was very critical of the XMRV identification- the sound evidence for viral involvement was emphasized. A logical-inference analysis showed that -most likely- a wrong viral candidate was chosen [1], Fig.1. Subsequent work has vindicated our first prediction (XMRV falsification), but additional work is required to strength the association with another candidate Virus that we propose: MFV (see later) [1]. Several studies have confirmed the *RNASEL*-HPC1 association [7] [8] [9] [4], but not all [10] [4] of them.

2.2. XMRV discovery

For another five years at the turn of the century, these discoveries on HPC-1 remained just suggestive of a viral involvement in PCA, for a locus -*RNASEL*- which behaves as a Tumor Suppressor Gene (TSG) -as already indicated by an interesting Editorial by Lengyel, in 1993 [11] and as suggested by others [12] [13]. Then Silverman with colleagues DeRisi and Ganem utilized a micro-array approach (*viro-chip*) [14], in order to try identifying the responsible virus [3, 15]. The first papers on XMRV appeared at the end of 2006/ beginning of 2007: they showed that XMRV was present at high frequency in patients homozygous for the R462Q allele (i.e., 8/20 or 40%) and that it is a xenotropic retrovirus with similarities with murine leukaemia viruses (MuLV) [16] [15]. Xenotropic retroviruses are endogenous viruses, which cannot infect cells of the original species, while ecotropic viruses do. Typically, endogenous murine retroviruses have been divided into two large families: ecotropic and non-ecotropic retroviruses [17] [18]. Ecotropic retroviruses -being still capable of active infection in the same species, i.e. mouse, cells- are present in only one or just a few copies (0-6) per genome. Their genetics is rather well clarified by several years of research [19]. The structure/genetics of the non-ecotropic retroviruses is more complex, also in view of the fact that they are present in a considerable (40-60) number of copies/genome. In recent years, particularly thanks to the work of J. Coffin and J. Stoye [20], non-ecotropic retroviruses have been clarified and subdivided into three subfamilies: xenotropic (XMP), not capable of replicating in-

side cells of the same species, polytropic (PMV), which are capable of replicating inside cells of several species including the original (mouse) and modified-polytropic (MPMV), which display altered properties in terms structure/function of the *env* gene [21] [17] [18]. The experiments, which distinguish among different subfamilies of non-ecotropic mouse retroviruses are: 1. infectivity/replication assays; 2. characterization of their structure by restriction enzyme and/or Southern blotting analysis; 3. complete sequencing [20, 21]. For a more detailed overview of this fascinating but rather complex scientific area, the reader is referred to two excellent review articles by J. Coffin and J. Stoye [17] [18].

2.3. Positive evidence

XMRV was also found integrated inside mesenchimal/stromal cells -rather than in tumour cell genomes- in proximity of genes of cell cycle or hormonal control, which could provide a reasonable link to carcinogenesis [16] [4]. Indeed, such mechanisms variably defined as “promoter insertion” or “insertional mutagenesis” appear to be the most likely involved in chronically (or non-acutely) transforming Retroviruses [22] [23]. This initial report by the discoverer group was followed up a few months later by another PNAS paper, by Schlaberg et al., in which XMRV was associated to approximately 23% of cases by immuno-histochemistry (IHC), while detection of viral DNA by PCR was quite lower (6%) [24]. Beside this rather surprising finding (since the opposite would be typically expected), this report also slightly contradicted the previous ones, since 1. XMRV was directly identified in the carcinoma cells and not in surrounding mesenchimal/stromal cells, 2. there was no evidence of an association between XMRV positive cases in PCa and RNase-L involvement by mutation/lower function, as previously described in the Urisman et al. paper [15, 24]. In that report, 40% of cases which were homozygous for the R462Q variant in RNase-L were XMRV+ [15]. In the following months of 2010, another group from Emory University in Atlanta (GA) also reported an association between XMRV and PCa, by employing three different and complementary technologies [25]: a) a very sensitive “nested” PCR assay, b) chromosomal fluorescence hybridisation (FISH) and c) very sensitive technology for detection of neutralizing antibodies (the same group and others had previously developed this technique for detecting anti-HIV antibodies) [26] [27] [25]. Also in this report, the serologic assay was the most sensitive, detecting XMRV antibodies in 27.5 % of cases (11/40), while positivity increased in carriers of the R462Q allele (8/20 –also in this study- or 40% of cases, which were RNASEL R462Q homozygous) [25]. Finally, this report confirmed, as in the original paper by Urisman et al., the presence of XMRV in stromal/mesenchimal and not in carcinoma cells [25]. In the same year, another group from Baylor College in Houston (TX) also detected an association between XMRV and PCa in 22% of cases [28]. However, virus was strangely detected in both tumour and normal cells of affected patients and there was no correlation –as in Schlaberg et. al - with RNaseL status [28].

2.4. Negative findings

Together with the appearance of such positive reports, however, a series of studies presenting negative findings started to appear in the literature. Many of these negative reports

came from European laboratories, although an initial negative study –often ignored– was from Johns Hopkins University (JHU) in the US [29]: see below. While the issue of XMRV detection in PCa was getting more controversial, another “XMRV-front” opened with the publication in October 2009 of a paper in *Science*, where Lombardi et al. reported detection of XMRV in 67% (68/101) of Chronic Fatigue Syndrome (CFS) cases [30]. While controls showed much lower detection rates, i.e. 3.7% (8/218), such value (as well as previous ones) was alarming, since it suggested that a few million people may be infected in the general “healthy” population in the US and probably elsewhere [31]. The initial Lombardi et al. paper was followed by larger numbers of negative reports, appearing in the months immediately after its publication: they will not be reviewed extensively in this chapter and the reader is referred instead to the NMD paper [1], with only one exception. In September 2010, Lo et al. published a PNAS paper describing rather frequent association between CFS and a retrovirus different from XMRV: indeed this virus appeared to be polytropic (P-MLV) instead of xenotropic (X-MLV) [32]. While some scientists applauded this novel discovery [33], the PNAS paper was accompanied by an editorial by Andrew Mason’s group, in which perplexities about these very findings were expressed [34]. Indeed, despite the relationship between the two viruses, it was extremely difficult to reconcile these findings or even to explain the discovery of XMRV as due to presence of P-MLV instead. In fact, the two viruses are clearly distinguishable by sequencing. Therefore, the idea presented at that time [33]: that the real culprit in CFS would be P-MLV and that the previous detection of XMRV should *de facto* be considered P-MLV detection, or that either virus could cause the same disease, was simply wrong.

The very first negative report for XMRV in PCa was from Hamburg, DE and was authored (1st) by one of the first co-authors of the original paper by Urisman: Nicole Fischer [35]. This suggests that very similar detection methods were employed in Germany: XMRV was detected only in one non-familial PCa (of 87) and one control (of 70) sample. Neither one of these cases was homozygous for the R462Q allele [35]. An even more striking negative result was obtained by Hohn and collaborators in Berlin [36], who did not detect a single positive case among 589 PCa patients tested: this study employed a sensitive nested PCR detection, RT-PCR for *gag* sequences as well as serology for XMRV-specific antibodies [36]. A number of patients (76) were studied for the RNASEL allele and 12.9% scored positive [36]. Similar negative results were published in additional studies from Ireland (139 cases) [37], Holland (74 sporadic cases) [38], Mexico (55 cases) [39], USA (over 800 patients from a collaborative effort between Baylor, Johns Hopkins etc.) [40] and UK (437 patients from UK, Korea and Thailand) [41]. In the last study, a few patients scored positive: for example 2 out of 6 of Thailand’s patients were positive, potentially reaching a score of 33%. However, evidence of contamination started emerging in this British International study: some of the amplified DNA did not contain a 24 bp deletion which is a hallmark of XMRV and other evidence suggested instead presence of P-MLV (as in the previous paper by Lo et al. on CFS) [41] [32]. A few assays, specific for contamination by mouse DNA, were therefore run to confirm identity of specimens. A very sensitive assay for Intracisternal A-type particles (IAPs) and mouse mitochondrial DNA was completely concordant with XMRV presence, clearly indicating

presence of contamination [41]. Therefore, this 2010 paper by Robinson should have already signalled a red-flag warning for XMRV research [41].

2.5. Strength of RNASEL – HPC-1 paradigm

At the International Congress on Muscle Fatigue in 2010, I strongly criticized the association between *PCa* and *XMRV*, on the basis of such negative findings, most of which had been already published in the literature (July 2010). My analysis at the congress extended to the technology employed, thus suggesting that the *viro-chip* assay was –most likely– the source of error [1]. Still, data on the *RNase-L* association with *HPC-1* were indicative of viral involvement. Contrary to the situation in *PCa*, in which a few independent reports confirmed XMRV presence, while they were contradicted by a limited number of studies, CFS association with this virus was essentially based upon the unique paper by Lombardi et al. in 2009, somehow overwhelmed by a plethora of negative reports [1]. However, also in CFS, the case for the likely presence of an infectious agent, most probably a virus, can be made. This is particularly clear, in view of the presence of “micro-epidemics”, often associated with CFS onset [1]. The rather strong evidence for a previous virus infection accompanied by the dramatic personal histories of CFS onset in thousands of patients could explain, but certainly NOT justify, the attachment of some patient-groups to the XMRV hypothesis, sometimes referred in the media as mass-hysteria [224]. We will later discuss whether the viral hypothesis should be completely dismissed in view of XMRV falsification or whether additional viral candidates should be investigated (see section 3).

2.6. XMRV controversy: looking back through 3 major Editorials

After 2010, the majority of XMRV reports documented negative results either in *PCa* or in CFS cases. Yet, the heated debate could have continued much longer, with some extreme defence of the XMRV hypothesis (J. Mikovitz) and with a more balanced overview of the criticisms by R. Silverman (see for example, his excellent review in *Nature Reviews of Urology*, extensively discussing criticisms) [4]. Examples of debates on possible infectious agents present in human cancers are abundant in the literature: for *PCa*, HPVs are still extensively discussed as potential etiological agents or onset-cofactors see discussion in Sections 4.3 (3) and 4.3.1 (c). What or who was capable of rescinding the “Gordian Knot” of XMRV cancer/CFS association? If we want to name a single scientist this is certainly John Coffin, although he extensively collaborated with other groups, especially with the group of S. Patthak. And yet, Coffin himself had written with J. Stoye in *Science*, accompanying one of the first papers on XMRV discovery –that of Lombardi et al. on the CFS association [30]– a positive editorial comment, which emphasized the future potential of such discovery [31].

- i. It may be instructive in this respect to re-analyse –so to speak: *after the facts*– the three major editorials, which accompanied the three major discovery-articles associated with XMRV. The first is the article by Dong et al. in *PNAS* at the beginning of 2007 [16], therefore immediately after publication of the Urisman et al. paper (December 2006). This article really gave credibility to the XMRV hypothesis, by showing that the virus was: 1. capable of replication in human cells, once a com-

plete copy of the provirus was cloned and reconstructed; 2. responsive to the IFN pathway, as it had been predicted in view of the RNase L mutations; 3. uses a specific receptor, XPR-1 (therefore capable of mediating entrance for both xenotropic and polytropic retroviruses) for infecting human cells; 4. in three cases analysed, XMRV was integrated in tumour cells in regions surrounding potentially interesting/important genes, in two cases next to transcription factor genes (CREB and NFAT) and in the third, next to a hormone response gene, causing inhibition of androgen receptor trans-activation (APPB2/PAT1/ARA67). The accompanying editorial, by retro-virologist Hung Fan, is certainly the most cautious and critical of the three editorials [43]. Although underlying the potential importance of these findings, Fan clearly indicated that they were generating more questions than answers and that only by answering such questions could the XMRV hypothesis be strengthened or proven [43]. In one sentence, his cautionary criticism was particularly evident: *“However, another possibility is that XMRV is not causal to PC, but reflective of the reduced antiviral status of RNase L QQ individuals; another novel virus whose sequences were not detected by the ViroChip might be the relevant agent”* (bold characters are my additions) [43].

- ii. The second fundamental paper for the XMRV hypothesis was the one by Lombardi et al. (2009), in which an astonishing 67% XMRV presence was documented in Chronic Fatigue Syndrome samples [30]. The paper was already briefly described, as well as the strong critical reaction it has generated, although this section is covered in more depth in the NMD review (see [1]) [30]. Surprisingly, the accompanying editorial written by John Coffin and Jonathan Stoye, appears to emphasize the positive aspects of these findings, rather than caution the readers about potential pitfalls, such as contaminations/artefacts [31]. It is apparent that the two Editorialists, among the major experts in mouse retro-virology, believed in 2009 that XMRV had strong connection to CFS, although it should be reminded that other viral infections have been previously associated with CFS (EBV, HHV-6, HTLV etc., see [1]) [31]. And yet Coffin's with Pathak's groups eventually *“put the nails into the XMRV coffin one by one”* [44]. Far from being a “changing party” episode, reassessment of scientific data and even of personal beliefs is an essential and intrinsic process of scientific endeavour. One of the greatest epistemologists of past century, Karl Popper, has identified in the process of empirical *falsification* one of the essential logical characters of science in western world. In his *“All Life is Problem Solving”* Popper suggests that our scientific theories develop as an evolutionary (almost *Darwinian*) process, in which it is however *falsification* rather than *verification* the discriminating instrument (*Occam's razor*). Therefore, it is just natural and physiological that today in science, hypotheses and theories are continuously re-evaluated and reassessed, although in this process strong intellectual honesty and courage are also needed. Most likely, in 2009 Coffin/Stoye positively reacted and were convinced by 1. the fact that XMRV demonstrated a clear homology to MLV endogenous sequences, but different enough and with constant/homologous difference (approximately 10% throughout the viral genome) to let us believe that this was a

totally new isolate. 2. The fact that all XMRV isolates detected showed strong homology among each other (less than 30 nucleotide variations in a genome of over 8000 bp.s), could be again evidence of an exogenous infecting agent (but also a contaminating virus). 3. Somehow, the general homology of XMRV with endogenous MLVs of approx. 90% may have been misleading still in 2009, since it might have suggested a mechanism of constant mutation accrual, as in phylogenetic analysis, of which the two editorialists are great experts [31]. In XMRV, however, recombination plays a major and determining role, as it was initially suggested in a PNAS editorial one year later, by Andrew Mason and colleagues (accompanying the third XMRV/MLV paper by Lo et al.) [34] [32].

- iii. Lo's paper initially appeared (or it was presented as) confirmatory of the infection hypothesis in CFS, since a murine retroviral sequence was detected in 86.5% of cases and only 7% of controls [32] [34]. The viral sequences however were not identical or very similar to XMRV, as previously reported, and appeared to be related to endogenous Polytropic retroviruses (PMLV). This generated some scepticism, as in previous work the viral sequences had little difference from the prototype retrovirus -XMRV. In his editorial, Mason underlines some discrepancies and yet does not clearly indicate that the finding of one xenotropic and one polytropic retroviruses are incompatible [34]. In other words, a general misconception could be –and apparently was- generated: there is an endogenous-like mouse retrovirus infecting cells in prostate carcinoma and CFS. In this scenario, *apparently* it didn't really matter whether it was marked with a P or with a X (for Polytropic and Xenotropic): the relevant and important point was that some type of murine endogenous-like retrovirus was infecting *Homo sapiens* in such disorders [34]. The paper by Ila Singh was also in line with such (mis-)interpretation [33]. On the other hand, as also pointed out in the previous editorial by Coffin and Stoye, the strength of the original XMRV hypothesis laid in the fact that all the isolates were similar to each other, although the prototype of XMRV appeared to be unique, different from any retrovirus known at that time [31]. Furthermore, Mason group's editorial suggested that, while the issue of which retrovirus exactly is present in PCa and/or CFS was being solved, a realistic and effective strategy could have been to test already potential therapeutic approaches with antiretroviral agents [34]. Again, such attitude is logically biased by the *caveat* that there was no firm evidence at that time for the real involvement of a retrovirus in both human conditions: this has been completely confirmed now by XMRV falsification. In fact, the paper by Lo et al. was rather good evidence *against* involvement of a retrovirus in both human conditions, since it suggested that contamination could be the cause [32]. Contamination, although denied in Lo's paper by a series of counter evidences, could explain the association with an endogenous murine polytropic retrovirus and, by extension, also with XMRV [32]. Andrew Mason group's editorial also emphasized the fact XMRV sequences appeared to be the result of recombinatory events [34]. They observed that in XMRV, while the 5' portion of its genome shares great homology to polytropic murine retroviruses, the 3' end is most similar to endogenous xenotropic MLV [34].

2.7. XMRV falsification

This observation, that inescapably leads to presence of recombination, was further developed approximately one year later in a seminal article by the groups of J. Coffin and S. Pathak [45]. In this Science paper in May 2011, Paprotka et al. convincingly showed that XMRV was generated by recombination during passage of the original tumor cells in nude mice [45]. The creation of human cell line 22 Rv1 was reported in 1999 after several passages by xenotransplantation, starting from 1993. The late passages /established cell line display presence of several copies of integrated XMRV provirus as well as high titers of virus production (10^{10} - 10^{11} PFU/ml). However, Paprotka et al. established a few essential and undermining criticisms: 1. First of all, fully infectious XMRV could not be detected in the original tumor explant (less than 1 copy/200 cells). 2. Second, two regions of strong homology with endogenous viruses could be detected: the 5'-end (called preXMRV-2) displays strong homology to PMLV endogenous sequences, while the 3'-end region (called PreXMRV-1) is most similar to an endogenous xenotropic retrovirus (XMLV). 3. Third, highly infectious "recombinant" XMRV started to appear in xenografts passaged in nude mice since 1996, i.e., three years after initial establishment of this tumour xenografts. This strongly suggests that infectious XMRV was created or has infected these cells between 1993 and 1996. 4. Fourth, the original nude mice strains utilized in xenotransplantation experiments did contain as endogenous viruses both the endogenous xenotropic virus (pre-XMRV-1, present in 6 out of 48 tested and typical of European mouse strains) as well as the endogenous PMLV (preXMRV-2, present in 25 out of 48 tested and typical of Asian mouse strains). 5. Fifth, the overall structure of the infectious XMRV could be explained by six recombinatory events between the two viruses: preXMRV-2 and preXMRV-1. Indeed, recombination is known to frequently occur during retrovirus replication, due to a polymerase (i.e., reverse transcriptase) switching between two different templates, therefore a mechanism of "copy-choice" as compared to the classical mechanism of "cut-and-paste" typical of general recombination [45] [46]. 6. Finally, the presence of a unique XMRV structure after so many recombinatory events strongly indicates that this "creation" occurred only once, most likely during xenograft passaging into nude mice [45]. The paper by Paprotka et al. therefore concluded the "XMRV Odyssey" with a most logical and well proven explanation and XMRV-falsification [45].

Additional evidence against XMRV as an exogenous virus infecting the human species were also obtained by the group of Jay Levy, who analysed some of the same CFS samples initially studied by Lombardi et al. Since these patients, initially reported as XMRV-positive, were found devoid of this retrovirus, this finding once more strengthened the evidence for contamination in positive samples [47]. A series of subsequent papers then reported evidence for contamination [45] [44] [48] [49] [50] in: 1. PCR reagents (even Taq polymerase) employed for XMRV detection; 2. microtomes or blades for tumours sections (even one year after the initial experiment); 3. contamination of several cell lines, beside the original 22Rv1. Prostate carcinoma cells lack the APOBEC-GA3 activity and are therefore susceptible to XMRV infection, while other human cells –for example human lymphocytes– appear to be highly resistant in view of the strong mutagenic activity of APOBEC-GA3.

3. MFV as potential candidate in PCa

Together with criticism of XMRV as potential candidate for CFS, we presented data in July 2010 [1] related to a novel viral candidate for both PCa and CFS: Micro-Foci inducing Virus or MFV. While the more specific aspects related to CFS association are presented elsewhere [1], MFV properties which link this virus to PCa will be here described.

3.1. Cancer Cluster Genetic Data

Micro-Foci inducing Virus was initially discovered in a paediatric tumor diagnoses-association generally defined as “*Cancer-Cluster*” (CC). A CC of neuroblastoma (NB) cases was diagnosed in Southern Louisiana in 1987-88 in the small town of Morgan City, while also the surrounding area appeared to be affected. A 12 fold increased NB incidence was recorded for a period of 18 months, while diagnoses then decreased to none [51]. This is a typical epidemiological behaviour of CCs, as it has been also recorded in other instances, such as paediatric leukaemia/lymphoma clusters [52]. Most of the tumours of this CC were conveyed to the Ochsner Foundation Research Center for further genetic analysis. The majority of them (66%) displayed elevated MYCN amplification, a well-known marker of aggressive NB. In one tumour with extremely elevated MYCN amplification (1000X the diploid value of controls), we started witnessing an elevated genetic instability in cultured tumor cells (see Fig. 1) [51]. This was accompanied by appearance of very small foci (Micro-Foci, MF) of rounded and refractile cells growing on top of the mesenchimal cells which typically grew up slowly and as monolayer in the initial tumor cultures (1ary cultures) [51] [53]. Furthermore, the initial dramatic amplification of MYCN seemed to disappear in growing primary cultures, apparently diluted out by the growth of mesenchimal flat cells (Fig. 1).

3.2. Isolation of MFV/MFRVs, partial cloning/sequencing

In order to find an explanation for this phenomenon, it was also noticed that the number of MFs was extremely variable, with some cultures having hundreds while others being devoid of them. An assay was therefore established by utilizing supernatants from cultures with hundreds MFs, with which we infected cells devoid of them. Since MF formation could be reproducibly transmitted even after ultra-filtration of such supernatants (through 100 µm filters), presence of a virus was hypothesized and confirmed by Electron Microscopy (EM). Transmission EM detected cytoplasmic particles of 65-73 nm for MFV (Fig. 2), while similar particles of larger size (85-92 nm) were identified in samples of paediatric lymphoma cases (MFV related Virus or MFRV), studied a few years later in Switzerland [51] [53] (Fig. 3).

Molecular cloning and partial sequencing of MFV/MFRV genome convincingly demonstrated that they share strong homology with members of the Reoviridae family, particularly Reovirus-3 (Dearing Strain) (Fig. 4).

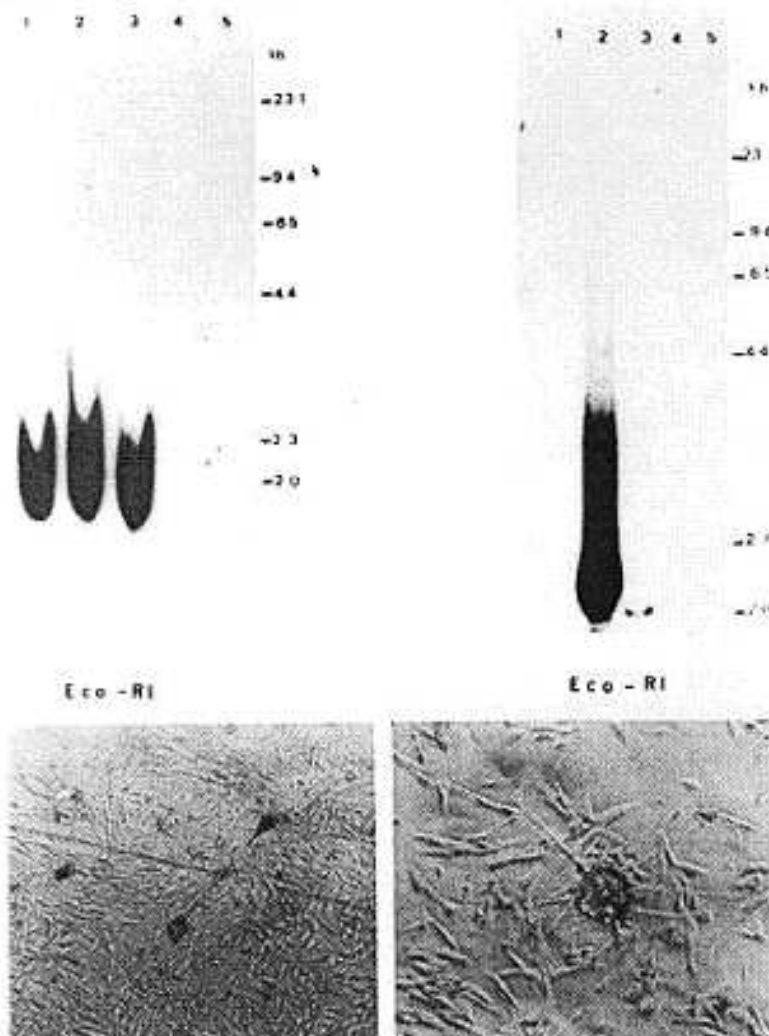


Figure 1. Top-left: Southern-blotting analysis shows high level of MYCN amplification in the original NB tumour from a Cancer-Cluster in Southern Louisiana. Lanes 1-3 contain DNA extracted from the original NB tumour, while lanes 4-5 two control DNAs (patient and normal blood donor peripheral leukocytes). Amplification was evaluated as 1000X fold by dilution experiments (not shown). Top-right: Southern-blotting analysis of DNA from the original tumour (lane 2) and from tumour cells passaged in culture for 2 weeks (lane 3) and 4 weeks (lanes 4-5). Bottom left: two microfoci, composed by small, rounded neuronal cells growing on top of a monolayer of large flat mesenchimal cells with Schwann cell markers. Lower magnification (40 X). Microfocus shown at higher magnification (100X).

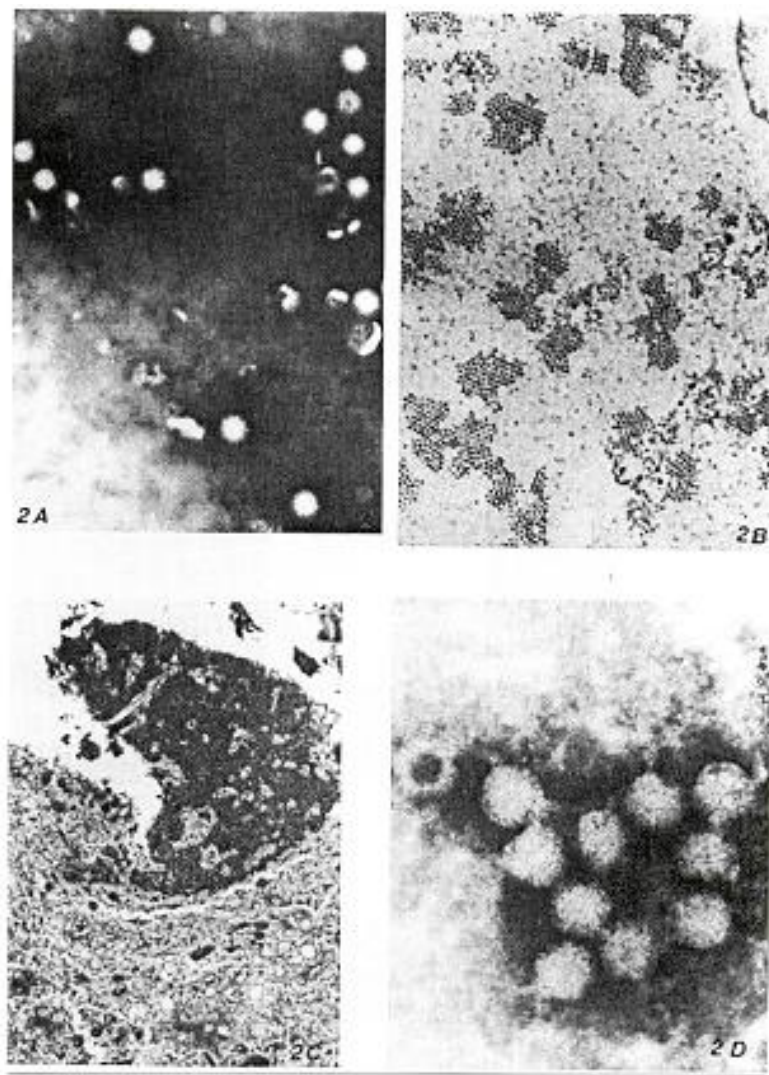


Figure 2. Electron Microscopy of MFV particles. 2A: negative staining of MFV particles (magnification = 100.000X). 2B and 2C: MFV viral "factories" in the cytoplasm of infected and transforming cells (magnifications: 15.000 and 10.000 respectively). 2D: Negative staining of MFV highest magnification (350.000X).

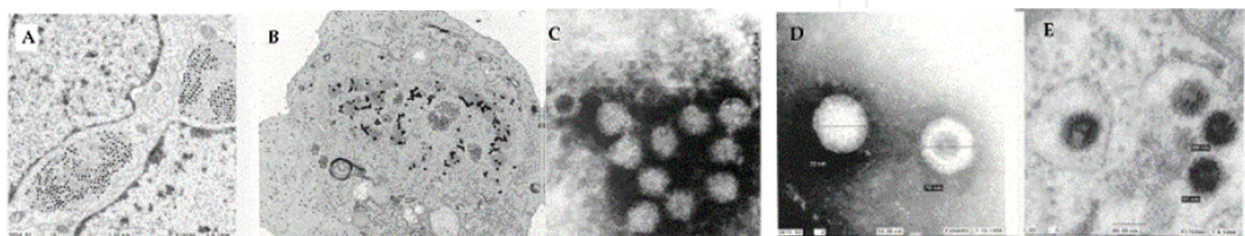


Figure 3. Electron Microscopy of MFV and MFRV particles. In 3A: MFV particles display a more localized pattern (15K X magnification), while in 3B, MFRV are spread through cell cytoplasm (5K X magnification). Fig.s 3C displays MFV at 350K X magnification (as in 2D) and Fig.s 3D-E MFRVs at 300K X and 175K magnification, respectively).

mfv-muns	1	TTGT GTAGTCACTAGGCCCCAAATGGAGTTAAGTCGAGCTCAGTCACTGAATGCCCAAAATGGAG	64
reo-3	1	TAGT GAAGTTACTAGGCTACAGATGGAGTTGAGTCGAGCTCAGTCCCTGAATGCTCAGTTGGAG	64
bl-virus	1	TGCTCAGTCACTGAATGCCCAAAATGGAG	28
mfv-muns	65	ACAGACGCCCAAATCGGCTCAATCATGTAGTCTGGATATGTATCTGAGACACCACACCTGCATCA	128
reo-3	65	GCGGATGTCAAGTCAGCTCAATCATGTAGTCTGGATATGTATCTGAGACACCACACTTGCATT	128
bl-virus	29	GCAGACGTCAAATCGGCTCAATCATGTAGTCTGGATATGTATCTGAGACACCACACCTGCATCA	92
mfv-muns	129	ATGGTCATGCTAAGGAAGATGAGCTTCTCGACCCCGTACGTGTCCCCTGACGTGAGAGAGAG	192
reo-3	129	ATGGTCATGCTAAGGAAGATGAATTGCTTGACGCTGTGCGTGTCCGGCCGGATGTGAGGAGAGA	192
bl-virus	93	ATGGTCATGCTAAGGAAGATGGGCTGCTCGACGCCGTACGTGTGCCCTGACGTGAGAGAGAG	156
mfv-muns	193	AATTATGGAAAAAGGAGTGAACTAAGAAGGGCTGGTGTGAACGCATTTCAAAGGAGTCACCT	256
reo-3	193	AATCATGGAAAAAGGAGTGAACTGAGACAAAGGTTGGTGCGAACGTATTTCTAAGGAAGCAGCT	256
bl-virus	157	AATTAGGAAAAAGGAG-GAA-TAAGAAGGGCTGGTAA	193

Figure 4. Comparison of sequences for Micron-NS –µNS- gene from MFV, a classical Reoviridae (Reovirus-3) and one isolate from Burkitt’s Lymphoma (BL). Divergence from Reo-3 is approximately 20%.

3.3. MFV-transformed cells growth *in vitro* and *in vivo*

Furthermore, extensive work *in vitro* and *in vivo* has convincingly shown that MFV causes malignant transformation in vitro and tumours in animals (see Fig.s 5-8) [51] [53].

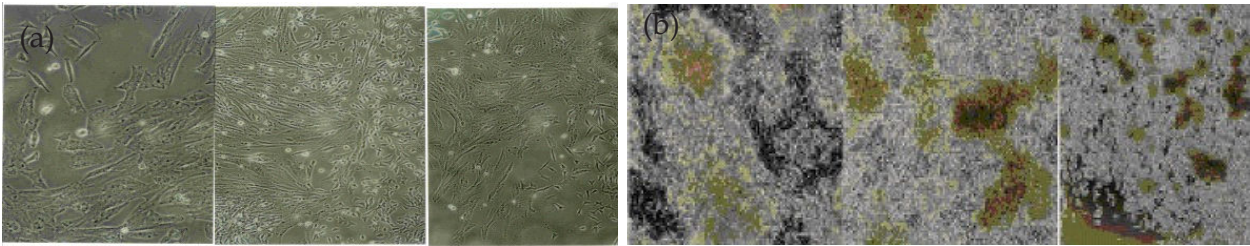


Figure 5. As shown in Fig. 5A normal, quasi-diploid SK-N-SH cells grow as mesenchymal cell (or Schwann-Cells) monolayers, but after MFV infection they transform (Fig. 5B) into aggressively growing NB cells. Transformed cells extensively grow in these *in vitro* conditions in the presence of low serum (2%), forming masses of rounded, small and packed cells (similar to MFs), which are loosely attached to the mesenchymal cell monolayer, often floating in the medium supernatant.

Fig. 5 shows the different patterns of growth of uninfected neuroblastic SK-N-SH cells (a) and MFV-infected/transformed SK-N-SH (b). While the original SK-N-SH cells grow slowly in low serum conditions (Fig. 6), MFV-transformed cells are undistinguishable in their growth properties from cells obtained from aggressive NB tumours -for example, SK-N-BE cells (Fig. 6).

3.4. Carcinogenesis Mechanism(s)

The molecular mechanism of carcinogenesis induced by MFV has been partially clarified when it became evident that normal non-tumorigenic diploid neuroblasts are rapidly destroyed by MFV infection: most monolayers are “wiped-out” in 36-72 hrs [54] [53] [55]. The only cells, which appear to sustain MFV infection without extensive apoptosis, have ampli-

fied the *MYCN* locus [54]. In Fig. 7, in the left panel, Southern blotting analysis (employing a *MYCN* specific probe) of the cell line *SK-N-AS* shows that the *MYCN* is diploid in mock-infected cells (-), but becomes highly amplified (approx. 100X) upon MFV infection and relative transformation (line 2: *SK-N-AS* +). A similar result was obtained with cell line *VA-N-BR* (3rd lane) [51]. Similar results were also obtained with cell line *SK-N-SH* (which is also initially diploid and non-tumorigenic in nude mice) by Q-PCR analysis. Upon MFV infection, these cells acquire a *MYCN* DNA level intermediate between the mock-infected cells (yellow, green lines) and cell line *IMR-32* (*MYCN* amplification approx. 20X: black line): *SH-10* cells (i.e., MFV-infected *SK-N-SH*) display an amplification level –by comparison- of approximately 10X (blue line).

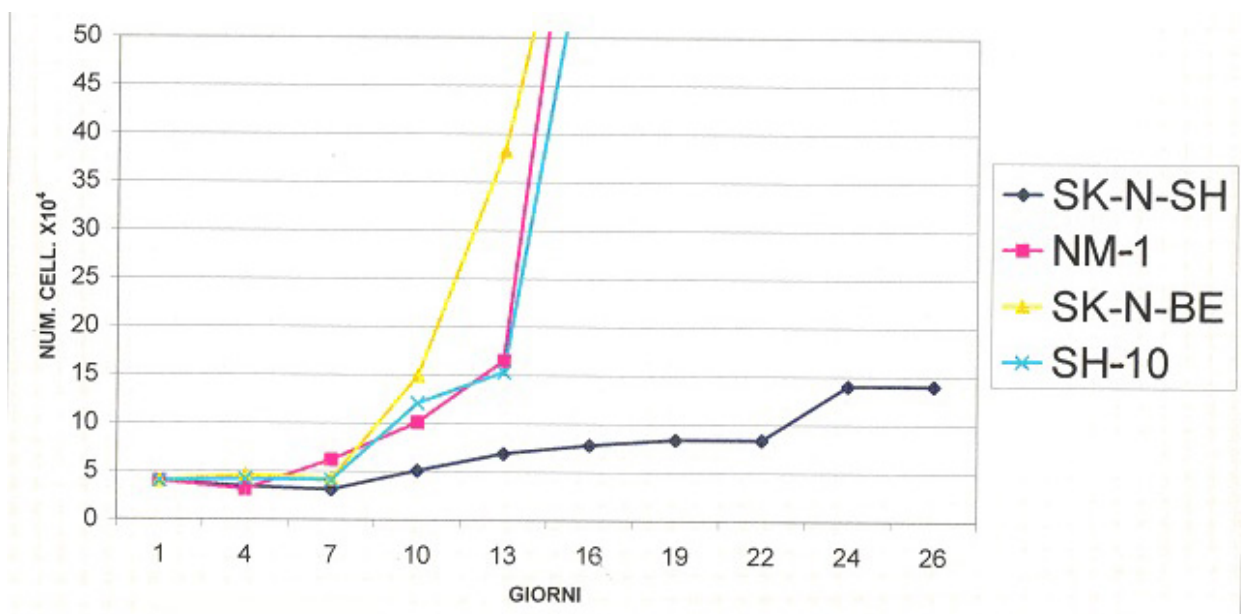


Figure 6. All cells were grown in Dulbecco's Modified MEM with the addition of 2 % Foetal Bovine Serum (FBS). NM-1 and SH-10 are two different clones of MFV- transformed SK-N-SH cells, while SK-N-BE is a Neuroblastoma cell line established from an aggressive tumour with *MYCN* amplification.

The same MFV-infected/transformed *SK-N-SH* cells -, shown in previous page [53]- were also employed in *in vivo* experiments of nude mice inoculation and relative tumour growth. Inoculation of MFV-transformed *SK-N-SH* cells into the left flank of a nude mouse causes

the appearance of large tumoral masses of NB cells (uninfected SK-N-SH cells were injected in the contra-lateral flank as control, Fig. 8).

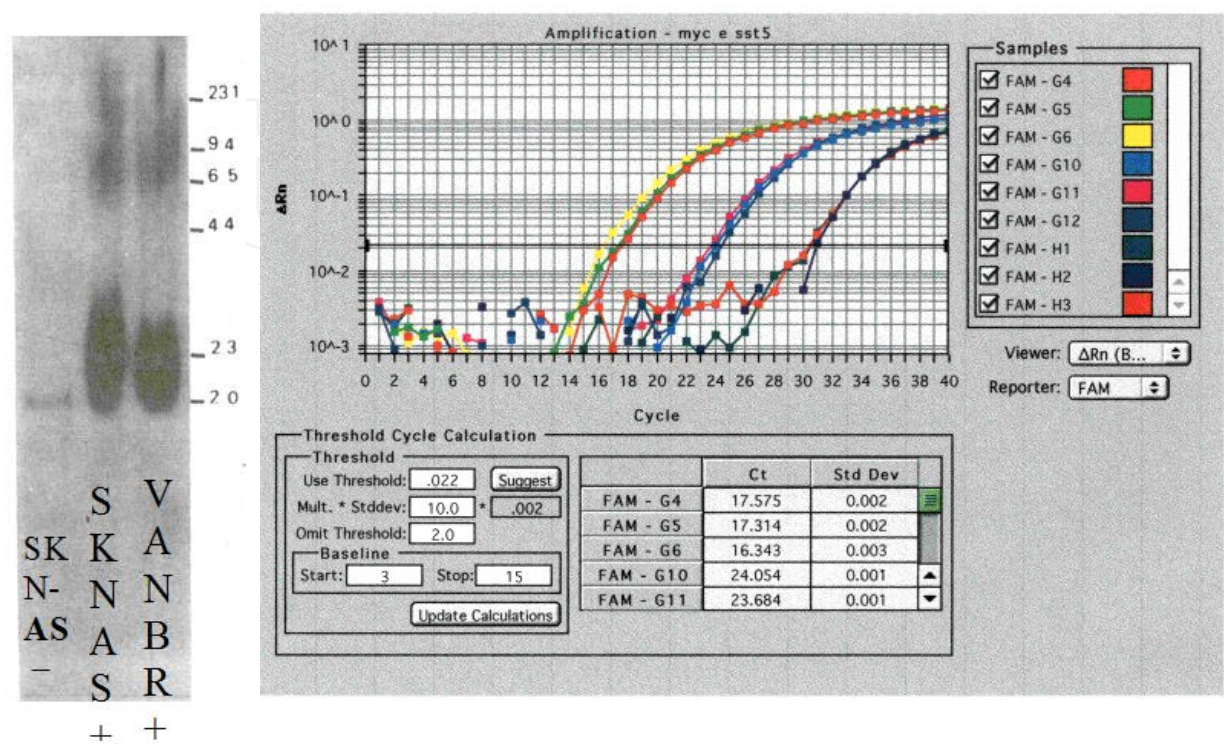


Figure 7. Southern Blotting and Q-PCR analysis of genomic DNA from cells infected/transformed by MFV. Left panel: Southern blotting analysis of cell line SK-N-AS before (lane 1) and after (lane 2) MFV infection; in lane 3, cell line SK-N-VR after infection/transformation with MFV (see also text description). Right panel: Q-PCR analysis of SK-N-SH cell DNA, DNA from SH-10 (the same line infected/transformed by MFV) and DNA from IMR-32 a cell line from aggressive neuroblastoma with MYCN amplification (approximately 20X). The relative level of MYCN amplification in SH-10 cells is estimated –by comparison– in the order of 10X.

4. Evidence for the association between MFV/MFRVs and prostate cancer

4.1. The Interferon (IFN) pathway

Evidence presented so far indicate that 1. in prostate carcinoma, an interferon-sensitive pathway appears to be affected. Attempts to identify an infectious agent (also on the basis of these observations), had led to identification of XMRV, a candidate virus, which has been eventually falsified by several groups (see part 1). However, as it has been emphasized in this chapter, evidence for viral involvement in PCa are rather strong and independent from the particular isolate XMRV. Indeed, as previously underlined, XMRV isolation is based upon usage of *viro-chip* technology and logical inference analysis predicts that this step is most error-prone [1]. In order to list and underline numerous elements indicating MFV as a strong candidate, the general IFN pathway is here considered and RNase-L as next point.

Although RNase-L is also an essential part of IFN pathway, it will be discussed separately, since it is prominent in view of numerous evidence and studies performed in PCa and other pathologies. Furthermore, the fact that transgenic animals *knockouts* for *RNASEL* gene do not develop tumours at higher frequency, suggests that additional elements in the IFN pathway may also be relevant [56] [57]. Since several years ago, the IFN pathways has been extensively dissected: beside RNase-L, two additional pathways are prominent: a) the PKR signal transduction and b) the Adenosine-Deaminase of RNA (ADAR) mechanism.



Figure 8. Tumorigenesis in nude-mice of SK-N-SH cells infected/transformed by MFV. Injection of 10^7 SK-N-SH cells (right flank) or SH-10 clone (left flank) with the same number of MFV-transformed SK-N-SH cells shows the out-growth 3 weeks later of large tumoral masses in the case of MFV-infected/transformed cells (SH-10). Histological analysis confirmed the presence of human neuroblastoma cells after xenotransplantation [53].

4.1.1. PKR

PKR was one of the best characterized pathways in the IFN signal transduction, starting from pioneering work of Isaacs and Lindenman, who initially characterized the IFN activity [58] [59]. One of the first enzymatic activities induced by IFN and its inducers (i.e., dsRNAs) is the dsRNA dependent Protein Kinase or PKR. PKR conveys the IFN message in several ways but especially by phosphorylation of: 1. PKR itself (autocatalysis); 2. the α subunit of eIF-2a ; 3. the inhibitor of transcription factor NFkB, IkB, thus releasing such inhibition; 4. the TAT transcription factor, essential activator of HIV; 5. the NFAT protein and 6. the phosphoprotein MPP4, which binds dsRNA and is activated during the M phase of cell cycle [58] [59] [60]. Among the different activities elicited by PKR activation, the best studied and known is certainly the inhibitory effect on protein synthesis (eIF-2a) [61]. Ser-51 Phosphorylation in this case blocks initiation of protein synthesis, by inhibiting the exchange of guanine nucleotide [61] [62] [63]. There are some discussions and discrepancies on the

regulation of PKR from different portions of the gene/structure: two knockout transgenic mice were generated by the groups of Karomillas/Bell [64] and Ch. Weissmann [65] [66].

The first one has been targeted in the carboxy-terminal of PKR, where is present the kinase activity, and doesn't show impairment of antiviral response or TNF- α responses, thus indicating the redundant role of PK activity [60, 64]. To the contrary, Weissmann's lab ko mouse and its MEFs are strongly inhibited in view of the deletion of the NH₂- portion of the protein, where the dsRNA-binding domain of PKR resides [65] [66] [60]. Effectors of PKR are several types of dsRNA molecules, both artificial and natural [67]. In this respect, the genome of MFV is a strong/ideal inducer of PKR in acutely infected cells, as we have documented in both mouse and human cells. Most likely, PKR induction also contributes to the strong apoptotic effects we have documented 36-72 hours post-infection [53]. In particular, MFV-infected cells completely block protein synthesis and strongly impair rRNA production (see later) and these effects seem to be mediated by PKR and RNase-L respectively. In prostate cancer, the same pathway of PKR appears to be downstream of another essential regulator and also Tumor Suppressor function of prostate cells: PTEN [68]. Therefore, typically the deletion of PTEN (which is extremely common in prostate cancer [69]) will lead to ablation of the TSG function of PKR by phosphorylation of eIF-2 α and block of protein synthesis [68] [69]. In view of the MFV/MFRV connection hypothesis, it is speculated that infection by this family of viruses will eventually cause/select for PTEN deletion, as this will inhibit cellular apoptosis, which would otherwise be inescapable [53] [55] [69].

4.1.2. ADAR

The additional and last form of IFN response here considered is the RNA-specific Adenosine Deaminase or ADAR, which is strongly induced after viral infection [70] [71]. Although such RNA editing was initially considered a rare phenomenon, almost a curiosity of RNA regulation and fine tuning, extensive genomic sequencing by NGS and high-throughput technologies have allowed to discern considerable editing in several DNA genomic sequences by a related Deaminase activity which targets DNA, called APOBEC, as well as ADAR activity on expressed mRNAs [72] [73] [74]. It is still not clear how efficient such mechanism may be at the RNA level, since ADAR activity may protect cells but also favour virus aggression or persistence inside infected cells [70]. DNA deaminase activity as well is still poorly understood, but new phenomena discovered in human cancer cells through Next Generation Sequencing (NGS) technology suggest that genome modulation and plasticity by APOBEC could also play a major role in carcinogenesis [73] [75].

Could the ADAR activity induced by Interferon (ADAR-1) be responsible for important antiviral effects in human and other cells? Although the question is still open and there are also examples of opposite regulation as previously mentioned –for example in Hepatitis Delta Virus (HDV) [70]–, important inhibitory effects were documented, with Measles Virus [76] [77] and with Influenza Virus in mouse cells [78]. Furthermore, a specific gene of the Adenovirus genome is responsible for counteracting the RNA-editing activity of ADAR: the VAI gene [79]. In the case of Reoviridae, little is still known but there is at least one animal model in which ADAR was induced by both artificial dsRNA and reoviridae genome

(which obviously is dsRNA), although the response modality of ADAR appeared quite different [80] [81].

Among human cancer as well very little is known, particularly in the case of prostate carcinoma [82] [73]. More data have been obtained in the case of brain tumours, since ADAR is known to effect important editing in brain neurons. In at least two tumour examples, hypoe-diting seems to characterize cancer cells. In *glioblastoma multiforme*, rectification of a mutated permeable glutamate receptor to Ca⁺⁺-impermeable receptor suppressed proliferation [83] [84], while in hypo-editing astrocytoma cells, re-balancing editing expression induced regression [85]. Additional work on ADAR/APOBEC in prostatic cancer and reoviridae is certainly warranted.

4.2. RNase-L, an essential pathway

The clearest evidence for viral involvement in prostate carcinoma and in human cancer in general was obtained through studies of the IFN response leading to RNase-L activation. The general scheme of IFN genes activation has been clarified through years of intense studies with several models, cell lines, laboratory and transgenic animals, in vitro assays and molecular/biochemical systems [60] [86]. Without getting into too many details –and referring instead the readers to some excellent review articles on this subject [60] [86] [87] [88] - two general types of IFN molecules are known: viral IFN and immune IFN. Both IFN- α and IFN- β (as well as IFN-omega) belong to the viral form, induced by viral infection [60], while the immune form is essentially composed by IFN- γ and is induced by immune stimulation. Focusing on viral genes, this is a rather large family in humans, since 13 genes for IFN- α , 1 for IFN- β and one for IFN-omega were mapped on the short arm of human chromosome 9 [89] [90]. None of these genes contains introns, while the only IFN gene with introns is the IFN- γ form with 3, on the long arm of chromosome 12. Two types of IFN receptors, IFNAR-1 and IFNAR-2 are known on human chromosome 21: they must heterodimerize for activation by IFN- α/β [91] [92] [93]. The following signalling is today understood mostly in the JAK-STAT transduction pathway, thanks to the work of several molecular biologists, first and foremost the group of Jim Darnell at Rockefeller University [94] [95] [96]. STATs are “signal transducers and activator of transcription” molecules: at least seven of them are known, i.e. Stat-1, Stat-2, Stat-3, Stat-4, Stat-5a, Stat-5b and Stat-6. STATs are activated by members of the Janus family of Tyrosine kinases (JAKs), of which 4 members are known, i.e. Jak-1, Jak-2, Jak-3 and Tyk-2. Various combinations of Jak’s and Stat’s elements are active in transducing both viral and immune IFN signalling [87] [88]. Additional important elements are the IRFs, (IFN Regulatory Factor) family [97], which cooperate in the activation of IFN-responsive genes. These are characterized by presence of regulatory elements: ISRE or IFN-stimulated Response elements [98], usually for viral IFNs and the GAS (gamma IFN activation sites) [99]. In conjunction with Stat’s, IRFs constitute the so called ISGF (IFN stimulated gene factor’s) [100]. Having clarified this terminology, ds-RNA activation of IFN pathway does not always or only use Jak-Stat elements. The transcription factor IRF-3 (IFN response Factor 3) acts as subunit of a complex called ds-RNA activated transcription factor complex (DRAF) by serine/threonine phosphorylation, translocation to the nucleus, associ-

ation with p300/CBP and gene activation [101]. Among the activated genes, the Oligo Adenylate Synthetase gene family, or OAS, is one of the most important, because it conveys the anti-viral signal to the next and final effector: RNase-L. In humans, three OAS genes of different sizes (proteins of 40-46 kDa -OAS1-, of 69 kDa -OAS2- and of 100 kDa -OAS3-) have been mapped on the long arm of chromosome 12 (12q24.2), thus suggesting genome duplications in *H. sapiens* [102]. Similarly to the effectors previously considered -PKR and ADAR-, OAS proteins do contain regions for binding dsRNA, the signalling and activating effector [103] [104] [105]. However, all three effectors contain separate regions with peculiar enzymatic activities: kinase, deaminase, synthetase [60]. The exact nature of dsRNA activators is not always completely clear, but hypothesized to be formed by or to contain dsRNA elements. In the case of interest, i.e. MFV/MFRVs infection, since these belong to Reoviridae family, it is clearly fragments or segments of viral genome (dsRNA) [53] [55]. Great variation has been documented in the extent/level of OAS activation and 2-5 A production [80].

Last element, RNase-L, is activated by 2-5 A signal molecules, typically oligomers with >2 elements for optimal induction, while RNase-L must dimerize for activation [106] [107]. This endoribonuclease is typically present as monomer in a latent form, essentially in every cell (tested so far), but -after 2-5 A interaction- it homodimerizes and is activated [107], although heterodimers with RLI (RNase-L inhibitor) have been also described. As previously mentioned, RNase-L gene, called RNS4, has been mapped on the long arm of chromosome 1 (1q25), on a location corresponding to the chromosomal site for the Human Prostate Carcinoma susceptibility (HPC-1) [108], as mapped with linkage studies by Jeff Trent and others [108] [6].

In view of the coincident chromosomal location of RNASEL and HPC-1 [6] [108] and the initial speculations by Lengyel and others that this gene may behave as a bona fide Tumor Suppressor [109] [12] [13], Silverman with DeRisi and Ganem undertook the described *viro-chip* high-throughput search for potential viral candidates, leading to XMRV isolation [15]. As previously described and discussed in Section 1, XMRV identification has been clearly falsified as a recombination artefact arising during xenograft transplantation in nude mice [45].

In view of the coincidental chromosomal localization of HPC-1 and RNASEL, what are the evidence for RNase-L involvement after MFV/MFRV infection? The acute phase of infection by these viruses is accompanied by a very high activation of RNase-L [53]. An assay, detecting ribosomal RNA (rRNA) degradation in infected and transforming cells was developed (U. Rovigatti, unpublished), thus confirming the extremely high levels of RNase-L induction, often leading to block of cell proliferation and apoptosis [53] [55]. In the past several years, groups in USA, France and Belgium have also documented a strong deregulation of this endoribonuclease in patients affected by Chronic Fatigue Syndrome. Pioneering work by Suhadolnick et al at Temple Univ. initially disclosed that 2-5 A activated RNase-L is upregulated in CFS patients [110] [111] [112]. This finding was followed up by description of a lower molecular weight form (37 kDa) of the same enzyme in CFS patients by the same group [113]. The French-Belgian group of De Meirleir et al. then showed that the 37 kDa fragment is proteolytically cleaved from the original enzyme of 87 kDa, by human elastase and/or cal-

pain [114]. The same authors also speculated that the levels of 2–5 A molecules with structures larger than dimers (trimers/tetramers) protect the 83 kD moiety from degradation [115] [116]. Presence of the 37 kD RNase-L could also explain the higher enzymatic activity (associated with the low MW form) and the ratio between the two forms was proposed as potential marker for CFS [117] [111] [118].

In conclusion, the extremely elevated levels of RNase-L in every cell type infected by MFV indicate that this could be an important parameter to be evaluated. Analysis is being performed for addressing the question of whether cells with impaired/mutated alleles of RNase-L (such as the R462Q allele) may be more resistant to the apoptogenic effect(s) of MFV/MFRV and could become better targets for carcinogenesis [53] [55] [1].

4.3. Inflammation - ubiquitous in PCa

Inflammation has been estimated as being somehow responsible for 20% of human cancers: these are typically linked to infectious agents, causing chronic infections as well as by other environmental factors [119] [120] [121]. While it appears to be an essential component of carcinogenesis –being defined as “the seventh hallmark of cancer” [122]– inflammatory processes are particularly prominent in PCa [121] [119]. Furthermore, inflammation in PCa adds an enigmatic component, which could be or become one of the best clue for deciphering its aetiology [55]. This enigmatic nature is however rather complex, as it can be distinguished by several elements:

1. The **paradox of a rather common disease** (most common cancer among men), afflicting this year over 300.000 people and killing more than 33.000 patients, only in the US [123]. For comparison, it has been observed that there is just a handful of cases described in the literature for Primary Seminal Vesicle Carcinoma, essentially in the same anatomical location [124]. This shows a peculiar and striking difference between two very close histological sites: PCa is diagnosed only in the prostate peripheral zone, rarely in the transition zone and almost never in the central zone [125]. This pattern is accompanied by typical phenomena of inflammation which is almost never of acute type (at time of diagnosis) and is characterized in the described zones by: a. chronic inflammation, b. benign prostatic hyperplasia (BPH), d. focal atrophy, e. a new type of inflammatory response defined as prostatic inflammatory atrophy (PIA) and finally developing into f. prostatic intraepithelial neoplasia (PIN) and/or g. prostatic carcinoma (PCa) [125] [126] [127] [128]. This pattern associates with an extremely common disease, the most common form of cancer in men, again suggesting a rather “common” causality [55]. As we will also consider other clues (see 2,3,4), the best explicatory mechanism is that of a common infection, in particular an infectious agent which is “endogenous” or “persistent” in *H. sapiens* and apparently much more frequent in certain human populations or races [129] [130] (see below)
2. **Variation in epidemiological data** for Chinese, Japanese or Arab men in comparison with the male population in Western Countries: for example, men born in South East Asia who then migrated to the US acquire a higher incidence within the first two gener-

ations [130] [131] [132]. In Shri-Lanka men, the incidence was recently assessed as 5.7 per 100.000 males, while such incidence rose up by twenty folds in immigrants to the Western Countries (for example, UK) [133]. This is again indicative of “life-style” rather than genetic factors being responsible for prostate carcinogenesis. Similar data are also present in Japanese men who migrate to the US [134]. Using similar epidemiological approaches, Hsing has recently divided different nations into three risk groups for incidence and mortality of PCa. The high risk group includes USA and several European Countries; to medium risk group belong European Nations such as UK, Italy and Spain, while Asian Countries are mostly included in the third, low risk group [130]. There is a general trend of increasing incidence, which the authors attribute to westernisation of life-style in the low-risk nations, but to TUR (trans-urethral resection) and PSA-testing in the high risk Nations [130, 134]. Interestingly, the incidence in US black males is 50-60 fold higher than that in Shanghai, China [130] [135]. The data by Julian Peto and others have confirmed these trends and the rapid changes in incidence/mortality in migrant populations (often within the first generation), thus emphasizing the concept that factors different from genetics (i.e. environmental, such as infectious agents) may be responsible [129].

3. The fact that inflammation, BPH and PCa **typically occur in the prostate peripheral area**, with almost no tumours in the central zone. This led initially LM Franks to hypothesize that in prostate cancer, the inflammatory effects are always accompanied by hyperproliferation and/or atrophy/necrosis [126]. Later, McNeal et al. have elaborated the same concept, by noticing in 1988 that in the whole gland, there is a clear-cut zonal distribution [125]. Out of 88 tumours studied, the majority (68%) arose in the peripheral gland, while 24% in the transitional zone and only 8% in the central gland zone. As mentioned, this suggests that infection through ascending urethra could be a responsible/associated factor [125] [136] [119]. Although bacteria have been initially suspected as responsible for inflammatory phenomena and as causes of carcinogenesis (*Neisseria Gonorrhoeae*, *Chlamydia Trachomatis*, *Trichomonas Vaginalis*, and *Treponema Pallidum*), such interpretation has been reconsidered in post-antibiotics era, since prostatic persistent bacterial infections have dramatically dropped [137]. Still, bacteria can be often grown even from expressed fluids of asymptomatic men [138]. The possible causality by viruses is still an open question, as extensively discussed in the first section on XMRV discovery and refusal. Among viruses, several have been extensively investigated throughout the years and particularly: **i.** *Cytomegalovirus* (CMV) has been investigated in view of its association with malignant transformation in vitro. Seven studies on tissues and 2 on serology do not support an association with PCa. [139] [140, 141] **ii.** *Epstein Barr Virus* (EBV) levels were shown to be not significantly different in PCa/BPH by Ab's, PCR and IHC; [142] **iii.** *HHV-8* was initially detected by Chang and Moore in *Kaposi Sarcoma* (KS) by subtractive hybridisation [143]. Initial positive results in PCa by Monini et al. [144] were later explained by infiltration with lymphocytes, most likely in *HIV-1* positive (and therefore *HHV-8+*) patients [145, 146]. Also, the strongly positive findings by Hoffman in men from Trinidad and Tobago can be probably explained by bias of selected controls [147] [148]. **iv.** *Polyoma Viruses* have been also associated with PCa: an ini-

tial report by Monini et al. [149] was followed by an interesting paper by Das et al., since they found *BKV* DNA positivity more frequently in malignant tissue [150]. However, also these studies were not confirmed [151] [152]. v. *Human Papilloma Viruses (HPVs)* : Extensive work has focused on these viruses throughout the years. Their relevance was also surmised from studies on women cervical/uterine cancer –pioneered by Harald zur Hausen (Nobel Prize for Medicine in 2008)- where *HPVs* are clearly involved in >90% of cases [153]. However: i) even Zur Hausen in reviewing this subject in his Nobel lecture dismisses the role of *HPVs* in PCa: in this sense, men would be some kind of “healthy carriers” of the viral carcinogen [154]; ii) Several studies have been published, some of which with positive results (for example, in an Argentinean study, 42% PCa were positive versus 0% BPH samples) [155]. However, in 24 studies from other Countries, there is no evidence of different *HPV* involvement between cancer specimens and controls [156]. Furthermore, the most recent meta-analysis didn’t show significant OR for PCa associated with *HPV-16* (OR = 1.09) or with *HPV-18* (OR = 1.08) infections [157]. Similar results were obtained by a recent review article (in press) [156]. iii) Another element which does not fit *HPVs* as potential carcinogens in PCa is the observation –initially made by Woodsworth [158] - that *HPV* infections do not elicit high inflammation or inflammation at all ([158], see also later discussion). As already mentioned, inflammation –most likely associated with a prostatic infection- is one of the Hallmarks of Cancer, particularly in this tumor type, PCa [122] [121].

4. Several reports (Platz; Mahmoud; Chan; Jacobs) have documented that **assumption of aspirin or Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)** for different periods could considerably lower PCa risk [159] [160] [161] [162]. These studies have been expanded in recent years, particularly by the work of Mahmud with several Meta-Analyses [163] [164, 165] [166] [167]. A positive correlation has been detected for aspirin usage (protection) with OR in the range of 0.81- 0.83 and this is confirming what was already known from animal studies, which typically display stronger and clear-cut protective effects (also with NSAID). In the case of NSAID, the effect is less apparent, or maybe diluted out [166]. In most recent nested case-control studies, Mahmud has confirmed a modest but significant effect with all propionates, ie. Ibuprofen, Naproxen etc., but not with other NSAIDs [167]. The question is somehow connected also to the relationship between BPH and PCa, since the former has been often considered a precursor and initial inflammatory response leading to the latter [168] [169] [170]. Additional findings however do not lend support to this hypothesis [171] [172]. Finally, a very recent study by Sutcliffe and others also does not show any effect for NSAID treatment in BPH as well as LUTS (Low Urinary Tract Symptoms) [173]. In either cases and also in view of the Mahmud meta-analyses, there may be a positive (protective) effect, but too small and/or diluted out [173].

4.3.1. How can this Inflammation-Scenario fit the proposed role of MFV/MFRVs

- a. First of all, this family of viruses infects the human population in the first years of life. By age 5, >95% of human population displays antibodies against Reoviridae and this

type of viruses have been shown to be capable of persisting in infected patients and animals for several months/years [174]. Furthermore and as discussed in the next section, MFV/MFRVs display all the features of a “Stem Cell Virus” (SCV), with features of interaction in early childhood with a developing immune system [53, 175]. That a prostate cancer stem cell may be present in PCa and targeted in the first phase of carcinogenesis has been longly hypothesized and recently confirmed [176] [177] [178]. Further studies are certainly warranted in order to assess presence of MFV/MFRVs in early childhood and during ontogeny [55].

- b. Different levels/types of MFV/MFRVs appear to be present in different human populations worldwide. However, a clear picture of the specific subtypes involved is still missing, particularly for what concerns MFV/MFRVs. This should be clarified experimentally (by viral nucleic acid and specific protein detection, presence of antibodies etc.) [174]. Furthermore, essential aspects of these viruses features are still missing as we do not have full knowledge of these viruses genome sequence/structure [53]. Patterns of infections and micro/mini-epidemics in different populations could be deduced and mirrored by what is happening with Rotaviruses (another member of Reoviridae) in the paediatric population, where dominance of one particular genotype was shown to dramatically change from year to year, at least in Central and Eastern Europe [179].
- c. The question of inflammation in PCa leads to the search of a causing agent in both affected patients and experimental systems. This chapter has dwelled trough different aspects of this essential question. The presented appraisal of potential responsible agents clearly indicates today a lack of credible candidates among bacterial infections. Even for viral candidates, the previous discussion showed that Herpes Viruses (CMV, EBV, and HHV-8), Polyoma Viruses and Papilloma Viruses lack some of the essential features as triggering agents [157] [156]. For all these viruses, extensive detection studies were performed for years without reaching any consensus nor obtaining evidence for their presence but in a limited percentage of cases [157] [156]. Same negative result was finally obtained for a Retrovirus, XMV, after much controversy and discussion [4] [45] [44] [48] [49] [180], while a previously retroviral candidate (HTLV-II) had been previously falsified in the case of CFS [181] [1]. It must be stressed that the essential feature discussed here is inflammation and the two most likely candidates in the previous list, i.e. Retroviruses and Papillomaviruses do not appear to induce inflammation as expected from analysis of PCa: I) Retroviruses are known to be capable of replicating inside cells, even without causing cytopathic effects or transformation for that matter [182]; II) for HPVs, the quoted work by Woodworth has clarified this point. He wrote [158]: “*A hallmark of HPV infection is absence of an inflammatory response. Basal cells express low levels of HPV early proteins, they don’t undergo lysis, and they are not rapidly recognized and destroyed by resident leukocytes such as NK cells and tissue macrophages.....HPV infections can persist and remain latent for long periods and may induce tolerance to HPV antigens..*”

To the contrary and as described in Section II, MFV/MFRVs are strongly apoptogenic and capable of inducing strong/very strong inflammatory responses in several experimental systems [53] [1]. In preliminary experiments, we have established primary cultures from ap-

proximately 20 cases of PCa. In the majority of cases, cultured cells displayed extensive cytopathic effects and did not survive for extensive passages, with three exceptions. While these results confirm previous descriptions by Frank, McNeal and by De Marzo's group [125] [126] [127] [128], they also suggest that whatever factor elicited strong inflammatory mechanisms in the prostate, with cycles of hyperplasia and of necrosis, the same factor may increase its effects during *in vitro* culturing [55]. We are presently testing this set of PCa tumors and relative cultures (different passages) for presence of MFV/FMRVs.

- d. Although never specifically tested, sialylcic acid and similar salts have been shown to be effective for the containment/ replication-inhibition of this family of viruses (reoviridae) [183] [184]. Any strategy or molecule capable of reducing their inflammatory responses would probably elicit similar results.

4.4. Stemness in PCa: MFV as a "Stem Cell Virus"

4.4.1. Prostate Cancer stem Cell or Cells ?

Essential aspects of PCa have been here discussed with emphasis on viral models [55] [1]. In this last section of the chapter and also in dealing with peculiar aspects of PCa carcinogenesis in connection with an infecting virus (with MFV/MFRVs as potential candidates), the issue of Cancer Stem Cells (CSC) or *stemness* will be discussed. The concept of Cancer Stem Cell dates back to much ground work in the past two centuries, with several pioneers such as Julius Cohnheim and Rudolf Virchow already in the 19th century: they predicted the existence of "*embryonic rests*" at the origin of tumor formation [185] [186]. At the beginning of XXth century, Pappenheim hypothesized the existence of embryonic stem cells, but it was only in the second half of '900 that experimental evidence was provided for them [187]. In Toronto, in the '60s and '70s, the research of Ernest McCulloch and James Till demonstrated that only a minute fraction of myeloma cells grew in *in vitro* assays in order to form colonies in semisolid media [188, 189]. The Toronto school settled the basis for further work by John Dick (see later). In the same years, similar work was carried on by Robert Bruce, showing that only 1-4% of lymphoma cells did transplant into recipients [190], and by Jim Griffin, who demonstrated low clonogenic potential for Acute Myelogenous Leukaemia cells growing in methylcellulose [191]. Three additional lines of research paved the way for the final development of CSC hypothesis. 1. Mutations or translocations were discovered in cells at birth, which became markers of leukaemia-precursor cells (i.e., TEL-AML1, MLL-AF4, AML-ETO, OTT-MAL): these cells behave as leukaemia stem cells, since they could differentiate into several lineages/compartments, while additional mutations were required for achievement of full-leukemogenesis [192] [193] [194] [52], 2. the work of Peter Fialkow clearly indicated clonal expansions of leukaemia stem cells in specific diseases such as CML, AML and Myelodysplastic Syndromes (MDS) [195] [196]. Most of this work was carried on using genetic markers such as G6PD, present on the X chromosomes: in females, one of the X is silenced by the so-called lyonization phenomenon (from Mary Lyon's work) [197], thus allowing to distinguish the expansion of individual clones in cases of heterozygosity (for ex., A/B alleles for G6PD) [198] [199]; 3. The work of A. Hamburger and S. Salmon in Tucson,

AZ, who also showed low frequency ($1/10^{-3}$ to $1/10^{-4}$) of colony formation from solid tumours [200, 201]. These experiments were, however only partly convincing or reproducible (for example, in S. Salmon's work) and further ethical questions and concern were raised by experiments of C. Sautham and A. Brunschwig who injected harvested cancer cells into the same cancer patients, again discovering that only large numbers (i.e., 10^6) were capable of tumor initiation [42]. Only at the end of the 80's and with the advent of automated high-speed Fluorescence Activated Cell Sorting (FACS) [202], the group of John Dick in Toronto was capable of convincingly and reproducibly demonstrating the existence of Leukemic Stem Cells (LSCs). This was accomplished by xenotransplantation assays, in which LSCs from AML were transplanted into Severe Combined Immunodeficient (SCID) mice, often crossed with Non-Obese Diabetic (NOD) mice, in which also the natural immune response (NK cells) is defective [203] [204]. In order to demonstrate stemness, these experiments had to prove the three essential features of stem cells, i.e. a. their capability of remaining dormant, b. their pluripotency, being capable of reproducing the full spectrum of cancer (i.e. leukaemia) phenotype; c. their capability of self-renewal by asymmetric division, thus, giving rise to both bulk tumour cells and their immature precursors [205] [206]. The paper by Bonnet and Dick in 1997 is considered the first clear-cut demonstration of the LSC concept by xenotransplantation [207]. Subsequently, the same concept (Cancer Stem Cell or CSC) was also proven in solid tumours, initially in breast cancer by Al-Hajj et al. in 2003, where the CSC was shown to be CD44+CD24-/low lineage [208]. However, additional markers were subsequently identified in breast cancer, one of the most interesting ones being Aldehyde Dehydrogenase (ALDH), which appears to affect the phenotype of cancer cells, being associated to capacity of detoxification and a more aggressive behaviour also in other types of CSC [209] [210]. ALDH however doesn't seem to be an universal marker, as it is not, for example, associated with a more aggressive phenotype in melanoma cells [211]. Another controversial issue in recent years has concerned the frequency of Cancer Stem Cells (CSC) in different tumours. For example, a recent paper by Quintana et al. calculated that with an assay employing NOD/SCID IL2Rg mice, up to 25% of melanoma cells were tumorigenic [212]. Similar controversies are also present for the identification of prostate CSC [176] [177] [178]. In fact, two different populations of SC and prospective CSC were isolated in PCa [213] [214]. An initial paper in Nature described regeneration of the whole prostate from a single basal cell, which in addition to classical markers of prostate cell differentiation (Sca-1+, CD133+, CD44+) also displayed presence of c-KIT receptor (CD117+) [215]. However, a subsequent paper by the group of Michael Shen convincingly showed that among luminal cells, rare precursors exist which display presence of the homeobox gene NKx3-1 in absence of androgens and are therefore called castration-resistant Nkx3-1 expressing cells (CARNs) [216]. These cells can reconstitute prostate ducts after transplantation and, upon deletion of the suppressor gene PTEN, rapidly form carcinomas *in vivo* [216]. Finally, the group of Owen Witte has recently shown that it is also a basal cell which can initiate tumorigenesis in nude mice through cooperation of AKT, ERG and androgen receptor [217]. It is therefore possible that more than one precursor stem cell is the target of malignant transformation in prostate cancer. Furthermore, this could also fit with the described PCa carcinogenesis, in which a rather diffuse "field effect" has been known for some time [218] [219].

4.4.2. Evidence for MFV as Stem Cell Virus, possibly involved in PCa Carcinogenesis.

- a. In initial preliminary experiments, we have shown that dilutions of MFV/MFRVs for several log.s (from 10^{-2} to 10^{-8} FFU/ml) will cause a similar number of transformants, thus indicating that the limiting factor was not the virus itself, but rather its target. Since an equal number of precursor stem cells are believed to be present in such cultures, it is hypothesized that the target is indeed a SC [1].
- b. The Micro-Foci induced by MFV have several features of deranged stem cells, in which genetic aberrations took place, such as MYCN amplification in neuroblasts and t(8;14) / t(2;8) in paediatric lymphomas (BL-type). Even the so-called organoids or tumorspheres of PCa (prostatospheres) have similar fetures of MFs: we are now performing experiments in order to convert normal prostate tissue/cell lines into prostatospheres by MFV infection [53].
- c. As mentioned, PCa is characterized by an initial oligoclonality, which underlines carcinogenesis through a “field-effect” (FE). Evidence of oligoclonality were also obtained by molecular biology studies (see next point). However a molecular explanation for FEs is still lacking [218] [219]: MFV/MFRVs could explain FE alterations in view of the slowly progressing infection, mostly through cell-to-cell contacts [53] [55].
- d. In approximately 50% of PCa, peculiar translocations TMPRSS2-ERG have been detected, which join together an androgen regulated gene: the transmembrane protease serine 2 gene, TMPRSS2, with at least 26 different genes for transcription factors [220] [221, 222]. Although data on association of translocations with PCa aggressiveness are controversial, the translocation is an excellent marker of clonality (individual breakpoints): they have shown initial existence of oligoclonal disease, further evolving into monoclonality during metastatic disease [223].
- e. We have shown in several experiments –and previously discussed in section 2- that MFV/MFRVs infection is associated or causing peculiar genetic aberrations such as MYCN amplification (I.E., Fig. 7) or t(8;14) / t(2;8) translocations in paediatric lymphoma [53]. Similarly, we hypothesize that the associated translocations induced by MFV/MFRVs in prostate cells are TMPRSS2-ERG translocations, which would confer resistance to virus-induced apoptosis [55]. Experiments are being carried out in several PCa biopsies already characterized for presence of translocations (in 25% of cases).

5. Summary and conclusion

In this chapter, a review of general literature, as well as data previously published or unpublished by the author, was presented with the specific aim of fostering an ongoing debate on prostate cancer aetiology. This debate was particularly spurred in the past six years by the controversy arising after isolation of a new retrovirus, highly homologous to endogenous xenotropic and polytropic murine retroviruses, called XMRV [55] [1].

The first part of the chapter has focused on XMRV, its isolation and eventual falsification, also as a “parable” of scientific trajectories and behaviours in science. The most heated episodes are probably missing (but the reader could easily find them in some well-written editorials, for example the one in *Science: False Positive*, [224]), but the scientific rationale should be easily followed from isolation to falsification. In this first section, I underlined the difference between RNASEL – HPC-1 association and XMRV identification. While the first is rather logically strong and corroborated by several evidence and years of research, the second was essentially based on just one high-throughput technology –kind of *shot-in-the-dark*- experiment. It is easily biased and prone to artefacts, as it happened in this instance. However, the idea of an infecting agent in PCa is strengthened by several other elements, of which RNASEL involvement is only one (also: IFN, PKR, etc are affected; presence of inflammation, involvement of peripheral prostate, field cancerization effects, etc.).

In the second part, the candidate MFV virus was presented, in view of its affinity with PCa (IFN involvement, RNase-L strong induction, generation of inflammatory mechanisms). For RNase-L, evidence was also coming from CFS studies, again pointing toward similarities between the two conditions (and cancer related fatigue –CRF ? [2] [1]). Furthermore, MFV was isolated from a cancer-cluster (NOT through PCR enrichment) in view of its strong/powerful biological activity. This is exemplified by its very strong apoptogenic mechanisms (entire cultures wiped-out in 36-72 hours) or its capability of inducing strong genetic instability, leading to genomic aberrations, such as MYCN amplification and t(8;14) or t(2;14) [53].

Finally, in the third section, the elements of PCa carcinogenesis, where MFV/MFRVs could show more clearly its effects, were underlined: they included IFN pathways, RNASEL, inflammation and MFV capability of infecting/transforming stem-like cells [53] [55].

What are then the MFV/MFRVs properties which should be emphasized or taken home as messages? Or how we should rationalize them in this ongoing debate on PCa carcinogenesis ? As mentioned, the RNASEL – HPC-1 paradigm is logically strong and also in CFS numerous evidence point toward infections (micro-epidemics, virus-infection symptoms, IFN pathway etc.) [3] [1].

One essential property of MFV/MFRVs is its biological power, which could lead to strong and persistent infections and long-lasting inflammations in affected hosts. This could easily explain cycles of necrosis/regeneration, which we witness in BPH, PIA, PCa [53] [119].

A second important question -not addressed by this review for limited space- regards the nature of these viruses and whether they have been isolated before. In view of the persistent/ long-lasting infections they can initiate, an easy comparison/association is with EBV, which infects *H. sapiens* in early childhood/youth (depending from geographic areas), then remaining latent, and has been also associated with lymphomagenesis and other human cancers. Indeed, in the *hospital-safari's* expeditions of Dennis Burkitt, there was a second type and non-Herpes virus (not EBV) constantly isolated [225] [226] [227] [228] [229] [230] [231] [232] (also: Jay Levy/ Thomas Bell, personal communications). All the data available today point toward a virus similar to MFV/MFRVs: in this sense and in view of our MFRVs data,

these viruses could be the missing link to malignancy in BL (EBV does not cause malignancy, it just immortalizes lymphoblasts) [53] [233].

A final question, in view of the close relationship of these viruses in terms of persistence in the human population, is what justifies this proximity, which –at least for its “cousin EBV”- resembles parasitism. Several authors and M. Greaves among them, have introduced elements of “*Darwinian-medicine*” analysis in our interpretation of carcinogenesis [234] [235] [52] [175] [236] [237] [53] [55] [1]. The *take-and-give* of MFV/MFRVs with *H. sapiens* infections could certainly be associated to some of their properties. For example, to their strong apoptogenic effects, leading to inflammatory reactions in BPH/PIA/PCa, but also possibly to useful tissue modelling/reshaping in other instances. The described strong relationship of these viruses with stem-like cells further suggests a closer partnership of MFV/MFRVs with *H. sapiens* in *Darwinian-medicine* terms. With all possible consequences.

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