1 TITLE: Biomarkers for detecting resilience against mycobacterial disease in animals

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Abstract:

Paratuberculosis and bovine tuberculosis are two mycobacterial diseases of ruminants which have a considerable impact on livestock health, welfare and production. These are chronic 'iceberg' diseases which take years to manifest and where many subclinical cases remain undetected. Suggested biomarkers to detect infected or diseased animals are numerous and include cytokines, peptides and expression of specific genes, however these do not provide a strong correlation to disease. Despite these advances, the basis for disease detection still rely heavily on dated methods such as detection of pathogen shedding, skin tests or serology. Here, we review the evidence for suitable biomarkers and their mechanisms of action, with a focus on identifying animals that are resilient to disease. A better understanding of these factors will help establish new strategies to control the spread of these diseases.

Introduction:

Resilience, in the context of health, can be defined as the capacity to overcome or recover from physiological challenges, be they infectious or otherwise. The health of an individual can start to deteriorate upon infection and progress further into ill health as the pathogen load increases. Pathology contributing to ill health can also be caused by the immune responses to eliminate the pathogen. Resilient individuals are able to reduce the pathogen load without exacerbating pathology and eventually recover (1).

A further complexity in the context of resilience to mycobacterial infections are pathogen survival strategies that enable them to remain dormant in the host and cause latent disease. In reality, it is difficult to definitively establish whether an individual is resistant or tolerant to a mycobacterial infection, in that the infection either does not establish or disease does not progress, or whether the individual has recovered from the disease. Sheep and cattle do recover from intestinal mycobacterial infection (paratuberculosis) (2-4) and some are resistant to infection (5, 6).

Resilience can thus be more broadly defined as the animal's ability to remain productive in the face of an endemic disease challenge, such as a mycobacterial infection. The ability to identify animals that have the potential to withstand disease progression in this setting is highly beneficial.

Paratuberculosis, a widespread mycobacterial infection of animals, is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a non-tuberculous mycobacterium which preferentially infects ruminants. MAP has been detected in food sources such as milk (7, 8) and the pathogen found in humans with immunosuppressive conditions such as Crohn's disease (9-11). While there is no proven causative association between MAP and Crohn's disease, it is clear that urgent research attention is required to find new ways to halt global spread of the disease in the animal population in order to prevent MAP from entering the food chain and reduce human exposure to this pathogen (12, 13). Current diagnostic tests including detection of the mycobacteria in faeces, or the presence of serum antibodies to MAP, are inadequate for definitive diagnosis, due to the intermittent nature of MAP faecal shedding and the low sensitivity of serological tests during early, subclinical infection.

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* is an important zoonotic mycobacterial infection of ruminants, with significant impact on agricultural production globally; Australia is the only major livestock exporting country to have eradicated bTB (14). The serious zoonotic potential and public health risk of bTB makes the swift identification and control of this pathogen in animal hosts and wildlife populations a key focus across human and veterinary research programs (15, 16). Issues with interference in diagnosis due to coinfection and cross-reactivity with paratuberculosis, the generally low sensitivity of currently available tests, and the spread and maintenance of *M. bovis* in wildlife reservoirs, have made eradication of bTB a difficult task (17). A final confounding factor in the diagnosis and treatment of veterinary mycobacterial infections is the presence of non-tuberculous mycobacterial (NTM) species. These bacteria include the *M. avium* complex (MAC) and the *M. terrae* complex which survive in environmental niches (18). NTM have

also been identified in fisheries leading to general and chronic mycobacteriosis, highlighting the widespread nature and the variety of mycobacterial species present in a range of environments (19). While mainly innocuous to livestock, simultaneous infection with NTM and either MAP or *M. bovis* creates further difficulty in the accurate diagnosis and delineation of disease, due to similarities between the antigens and cross-reactive host immune responses (20-22). In this situation, disease-specific biomarkers may provide an alternative to current diagnostic techniques such as the tuberculin test or serological tests.

Both paratuberculosis and bTB have recently been ranked as the second most significant infectious veterinary disease in food producing animals and zoonoses respectively (23). It is therefore evident that mycobacterial disease detection and management within animal populations must be improved, and while resilient animals may play a key role in reducing mycobacterial diseases, the accurate identification of such individuals is paramount to future efforts. New ways of distinguishing animals that are resilient, or susceptible, to disease will provide new strategies for managing the spread of disease. This has led us to consider the literature on other biological markers that could be useful in the diagnosis and control of these diseases.

Biomarkers of disease are objectively measurable indicators of normal and/or disease conditions, which must be highly specific and sensitive to accurately denote disease (24). As a diagnostic tool, biomarkers not only indicate the presence of disease, but may also differentiate between disease states, treatment efficacy and outcomes. In order for a biomarker to be considered acceptable and reliable, it must be both sensitive and specific for the appropriate disease or disease state (25). Ideally, biomarkers should also be from samples which are collected easily by minimally-invasive methods and use measurement technologies that are readily available in diagnostic laboratories (26). The possibility of prognostic biomarkers to demonstrate the likelihood of, and resilience to, disease have promising applications to aid in the management and control of paratuberculosis, and possibly that of bTB.

The chronicity of mycobacterial diseases and the spectrum of disease outcomes makes it necessary to definitively characterise the disease 'phenotype' being detected by any biomarker test. For example, using an experimental infection model for paratuberculosis in the natural host, we have shown that even resilient animals can shed MAP in faeces for a limited time when young (5). To this end we have recently published a guide to characterising the spectrum of disease outcomes in ovine paratuberculosis (27) which will be useful for researchers interested in discovering biomarkers to identify specific disease outcomes. An additional benefit of characterising protective immunity using biomarkers is that it can also be used to guide better vaccine design. Regardless of the vaccine formulation, ultimately the ability to mimic processes that overcome natural infection will provide effective protection against disease.

A range of novel biomarkers have been suggested for mycobacterial diseases, ranging from host immune proteins and molecules, including cytokines (summarised in Figure 1.), as well as differentially expressed miRNAs and genes. Current biomarkers for paratuberculosis are primarily related to the identification and diagnosis of disease, however as TB-associated biomarkers have demonstrated the ability to discriminate between active and latent disease while also functioning as prognostic markers (28-31), there is potential for paratuberculosis- and bTB-specific biomarkers to detect "silent", subclinical infections and to identify disease resilient animals.

Immunological biomarkers

Antibodies

Although the role of the humoral immune response in host immunity to intracellular mycobacterial pathogens is not fully understood, it is recognised that specific antibodies are detectable in the serum and may be important in protective immunity (32-34). Serum and milk antibody ELISA assays are common diagnostic tests for paratuberculosis, although less commonly applied in bTB. Current

commercial test methods for paratuberculosis have highest diagnostic sensitivity in the later stages of disease when animals are infectious, with low sensitivity to detect early disease (35). However, in an experimental challenge model in sheep, animals that were classified as resilient to disease, as lambs, had a stronger antibody response than those where disease progresses (5). This offers potential new applications for serological tests to be used during subclinical disease stages to identify resilient animals.

The isotype of antibody detected, as well as the antigenic target, can impact serological test efficacy. A range of antigens have been tested in an attempt to improve early disease detection in both paratuberculosis and bTB (36-38). Immunoglobulin (Ig)G antibodies are the most common isotype used for mycobacterial antibody ELISAs, however targeting different isotypes may be more informative. A recent study has shown that circulating *M.bovis* antigen in association with IgM was present in the serum during the early stages of infection (39). IgA, the main isotype present in mucosal secretions, has also shown potential for identifying resilience, being associated with protective responses in TB (40). An investigation into MAP-specific faecal IgA immunoglobulins has found that these antibodies can be detected during paratuberculosis disease progression, but this is transient and appears to be related to environmental MAP load (41).

While not as well-understood as cell-mediated responses to mycobacterial infections, antibodies are clearly indicative of exposure to pathogens and disease states, and may yet play a key role in defining phenotypes and resilience to mycobacteria.

Cytokines and chemokines

One of the key immunologic responses characteristic of mycobacterial infection is the elevation in IFN-γ secretion, however the application of IFN-γ as a diagnostic cytokine is limited as it is an indicator of exposure rather than disease *per se* (5, 42). There is potential for this cytokine as a

biomarker of resilience in sheep as these animals have a higher early IFN-γ response when young (5, 43). A range of other cytokines and chemokines have been reported as differentially regulated between infected and uninfected populations (summarised in Table 1.), as well as between active and latent TB states, and these are likely applicable to other mycobacterial infections. These potential biomarkers warrant further investigation, although there is a lack of consistency across studies as to the degree and nature of cytokine expression, possibly due to differences in cell type assessed, stimulating antigen, and experimental techniques used. Activated T cell and related cytokines including, but not limited to, IL-2, IL-3, IL-6, IL-7, IL-8, IL-9 and IL-10, have been reported to differ significantly even within infected and healthy control groups in studies of human mycobacterial infections (44-49). It is evident that further investigation, especially regarding pathogen-specific responses, is required to determine if cytokine profiles can accurately detect and differentiate between disease states.

Variations in cytokine signatures in active versus latent mycobacterial disease have also been demonstrated, with cytokines such as TNFα, IL-12, and IL-17 reported to be more abundantly expressed during active tuberculosis infections compared to latent infection (50). More recent investigations into cytokines as biomarkers and discriminators of active versus latent infection have suggested that combinations or ratios of multiple cytokines are more efficient at categorising disease than a single biomarker. One such combination with promising diagnostic potential are IL-2 and IL-10, detecting not only disease in TB patients, but also distinguishing between active and latent infection (49). With IL-2 ligation activating JAK-STAT signalling and regulating T cell responses, and IL-10 acting as a key immunosuppressive cytokine, the combination of the two could prove to be a major indicator of mycobacterial disease. Multiple studies have also proposed the combination of IL-2 and IFN-γ and their respective levels as a diagnostic marker of latent TB infection (49, 51). *Ex-vivo* studies of TB have also yielded possible combinations of predictive biomarkers, and cytokines that act as correlates of treatment success. Firstly, increased expression of IL-4 and its antagonist IL-

163 4δ2 during treatment, and subsequent changes of the ratio between the two, have been reported to
164 be indicative of disease outcome, with lower IL-4 and IL-4δ2 linked with better treatment outcomes
165 (52). Similarly, the ratio of IFN-γ and IL-10 may also be indicative of treatment success in TB patients.
166 IFN-γ characteristically increases during infection and IL-10 decreases, in keeping with the need for
167 strong T cell responses to control an intracellular pathogen. Low ratios of IFN-γ and IL-10 were
168 observed in early infection, and subsequently improved during and after treatment, indicating that
169 this may correlate with treatment efficacy (53).

170 Cytokine profiles during MAP infection in both sheep and cattle also provide possible
171 biomarker targets. These include cytokines such as IL-10, IL-12p40 and IL-3 as they are often
172 associated with different disease pathologies in paratuberculosis (54) (Figure 2). IL-18 and similar Th_2
173 related cytokines are symptomatic of specific pathological lesion types in bovine MAP infections
174 (55); while an increase in IFN-γ, osteopontin, and IL-17 may suggest a shift towards a Th_{17} response
175 in MAP infections (56). A similar range of T cell cytokines and chemokines including IP-10, IL-22 and
176 IL-17A have been suggested for bTB, however as with paratuberculosis and TB there is no widely
177 accepted or employable signature (57-59). Originally called CXCL10, IP-10 was first described for its
178 chemoattractant properties and role in the recruitment of T cells to sites of inflammation, but has
179 been identified as a possible biomarker of infection in TB and bTB with the potential to differentiate
180 between latent and active disease (60, 61). IP-10 is currently one of the most promising chemokine
181 biomarker candidates for bTB, with evidence of a specific response to *M. bovis* which correlated
182 strongly to the production of IFN-γ, further suggesting that the combination of cytokine and
183 chemokine biomarkers may be more applicable than single marker measurement (62). As IP-10 has
184 also been shown to distinguish between cultutre positive and culture negative *M. bovis* samples, this
185 biomarker can potentially provide a rapid alternative to traditional culture diagnostics for bTB (63).

186 Studies profiling the chemokine immune responses in pathological presentations of
187 paratuberculosis and bTB have often found contrasting results and patterns of expression, and could

have been influenced by differences in experiemental design including *in vitro* or *in vivo* conditions of the study (54, 57, 64-66). Suggested cytokine and chemokine biomarkers for each stage of disease and pathologies are summarised in Figure 1. Due to the granulomatous nature of mycobacteria, chemokine recruitment of leucocytes may be a host response to contain the invading bacteria, and the restriction of this process by mycobacteria may act to subvert the host immune response and establish a latent infection. Downregulation of key chemokines such as RANTES (CCL5) and monocyte chemoattractant protein 1 (MCP-1 [CCL2]) in paratuberculosis could provide alternative biomarkers for diagnosis alongside IFN-γ assays . To date, there has been no discernable pattern of expression of significant chemokines such as CCL3, CCR, and CXCL11 between disease pathologies of paratuberculosis and bTB, suggesting the immunological response may be too variable and individual specific to function as accurate and repeatable biomarkers across differing populations (67, 68).

Although these combinations require further validation across animal breeds, sample types and mycobacterial species, their role as indicators of disease in MAP and *M. bovis* infected animals may prove to be valuable in rapid, reliable and simple detection of disease with improvements in diagnostic technologies.

Transcriptomic biomarkers

Many studies have investigated gene expression in paratuberculosis and bTB pathogenesis, resulting in a long list of differentially expressed genes for these diseases, and are summarised in Table 2. Key functional pathways such as antigen presentation and MHC processing and lipid metabolism are altered during mycobacterial infection (69-73). Genes from these pathways may yet provide key resilience or susceptibility biomarkers in MAP infection.

Among the differentially regulated genes with potential as diagnostic biomarkers in mycobacterial infections are *Tfrc*, which encodes the transferrin receptor, and *LTF*, which regulates lactoferrin; they are often attributed to the pathogen's metabolism of host iron via the action of mycobactins (74-76). Similarly, *S100a8* and *S100a9* are differentially regulated and have been proposed as biomarkers for comparable inflammatory bowel diseases (75, 76). Together, the S100a8/9 proteins form the heterodimer calprotectin, a biomarker for inflammation which leads to inflammatory responses and immune cell migration and has been detected in MAP lesions, suggesting these genes play a role in disease pathology (77, 78). Haptoglobin, controlled by the *Hp* gene, is an anti-inflammatory agent that not only disrupts neutrophil and phagosomal activity, but also disrupts bacterial iron sequestering. This response is thought to be a result of the host's immune system limiting the harmful immunopathology of MAP infection. Matrix metalloproteinase 9 (*MMP9)* and its inhibitor *TIMP1*, are both upregulated during paratuberculosis and TB and are documented as consistently up-regulated genes in TB (74, 75, 79). Two β-defensin genes have also recently been shown to be up-regulated in MAP, *Defb1* and *Defb10,* indicating that their antimicrobial and immunomodulatory role may be indicative of host responses to bacterial infection (74). Along with this gene subset, Th1 chemokine genes such as *CCL4, CCL5, CXCL9, CXCL10* and genes related to metabolism including *IGF1* and *TCF7L2*, are up and down regulated respectively in paratuberculosis (76). A novel biomarker signature has been established from these differentially regulated genes in early MAP infections. Combinations of these 8 genes (*Timp1, MMP9, Hp, Tfrc, Defb1, Defb10, S100a8,* and *Serpine1*) have been demonstrated as potential biomarkers of various disease and exposure states of paratuberculosis (74) (Figure 2). Differences between case definitions and disease classifications between studies does however make comparison difficult, and supports the need for standardised practices (27). Although this is extremely promising for disease detection and as biomarkers for paratuberculosis, further validation in both laboratory and on-farm settings must be undertaken before their potential for identifying resilient and susceptible animals is confirmed.

In a similar manner to human TB and paratuberculosis, early gene expression in bTB correlates to the immune response and pathology with an early increase in Th_1 cytokine related genes, and a switch towards Th_2 cytokines as infection progresses. A panel of transcriptomic biomarkers have been suggested including the chemokine genes *CXCR3* and *CCL1* and TLR2/4 genes, along with *TNF, BCL2, NFKB1, IL16, IL8, EEF1G, ADAM17, IER5, PHB2, STK17B, CD84, CD81, MCL1, TBK1, ATK1, PRKCB1,* and *RPS6KB2* (80). While this panel is predominantly protein binding and transcription related genes, it displays the trend of immune suppression by mycobacteria and *M. bovis* and may provide an alternative to the current immune based diagnostics used in bTB identification.

Protein biomarkers

The analysis of circulating proteins and serum proteomes has also yielded promising candidates for biomarkers in MAP and other mycobacterial infections (Table 3). Mass spectrometry has detected a number of proteins either over- or under-expressed, with some, specific to MAP infection (81). Studies assessing both early and late stages of mycobacterial infection have shown a dysregulation of several pathogenically significant proteins including vitamin D-binding protein, a potential biomarker for general mycobacterial infection, found in both paratuberculosis and bTB (81-83). As vitamin D is involved in macrophage activation and is a known anti-tuberculoid agent acting via TLR signalling pathways, its expression in paratuberculosis may be attributed to the immune response in the early stages of infection. Glycoproteins, proinflammatory fetuin, alpha-haemoglobin and serine protease inhibitor are also differentially expressed proteins in both bTB and paratuberculosis, acting as biomarkers for general mycobacterial diseases in animals (81-83).

Proteomic analysis of serum proteins of MAP infected cattle has yielded further possible specific biomarker targets, such as complement proteins, actin binding proteins, and clotting factors

261 associated with thrombin and fibrinogen (84). These proteins of interest, along with their
262 corresponding coding genes may provide diagnostic biomarker signatures. Transthyretin and retinol
263 binding proteins have been identified as MAP-specific biomarkers. Vitamin A (retinol) is involved in
264 the maintenance and differentiation of immune cells. It is transported by the negative acute phase
265 protein transthyretin, which may be an indicator of early disease (82, 83). Transthyretin is also an
266 indicator of malnourishment in diseases such as HIV and cancer and may show similar changes in a
267 chronic wasting disease like paratuberculosis. Cathelicidin is specific for advanced MAP infection,
268 possibly related to a shift in the bacterial response to induce shedding and escaping from
269 macrophages, or a host antimicrobial control response (82). Investigation of the proteome may
270 provide potential pathogen protein biomarker candidates, however the homologous nature of
271 mycobacteria and issues with cross-reactivity mean that this requires much greater research and
272 validation. Preliminary research into identifying specific proteins from the secretome has provided
273 promising novel antigens as serodiagnostic biomarkers, although further investigation must be
274 undertaken (85).

275 Other suggested bTB protein biomarkers include the host proteins alpha-1-antitrypsin, alpha-1-
276 antiproteinase, and fetuin-A and the pathogen proteins ESAT-6, CFP-10, MB2515c, and Pks5 (81, 82,
277 86). Advances in protein array chips and mass spectrometry technologies will allow discovery of
278 other biomarkers using pathogen proteomes and circulating peptides in the future.

279

280 ## Extracellular vesicles

281 Extracellular vesicles (EVs) include exosomes, microparticles and apoptotic vesicles and are key
282 cellular transport and signalling entities. The importance of these vesicles was originally
283 underestimated, believed to be waste disposal units removing cellular debris during reticulocyte
284 maturation (87). Both exosomes (<200 nm) and microparticles (<1000 nm) are now prime targets for

targeted drug delivery and gene therapy, with several technologies for their use in the treatment of major human diseases in development (88-90).

Exosomes are released from multivesicular bodies following fusion with the plasma membrane and are formed through a series of endocytic events. Following their formation, multivesicular bodies fuse with the plasma membrane and release their cytosolic endosomal bodies, which become exosomes once liberated (91). In comparison, microparticles (also known as microvesicles and ectosomes) are formed and released via budding or 'blebbing' of the cellular membrane. This is a steady state process which may be upregulated following stimuli such as infection and include specifically enriched cargo for biological communication. Both exosomes and microparticles contain a range of enzymes, proteins, and RNA molecules, and have several functions, often highly dependent on the constituents and therefore their cell of origin (Figure 3).

Vesicles transport mycobacterial products such as lipoarabinomannan and phosphatidylinositol mannosides, which are contained in, and released from mycobacteria-infected macrophages through EV secretion. The shuttling of both bacterial and viral components further supports the role of exosomes in immune surveillance and intracellular communication (92). These EVs secreted from macrophages are able to stimulate a pro-inflammatory response, triggering the release of TNFα, nitric oxide, and the chemokine RANTES (93-95), as well as transferring mycobacterial RNA and ultimately effecting infection outcomes (96). Similarly, EVs secreted from host neutrophils appear to work in favour of the immune response and promote clearance and mycobactericidal activity (97).

Extracellular vesicles may prove to be extremely useful vaccine candidates and diagnostic or predicative biomarkers for mycobacterial diseases such as paratuberculosis and bTB. Their stability and circulating nature, as well as their ability to be isolated from minimally-invasive biological samples such as saliva, urine and blood make them prime targets. Differentially expressed proteins and molecules contained in vesicular compartments may also provide useful markers for treatment

efficacy and indicate disease resilience to mycobacterial infections. A small number of studies have identified *M. tuberculosis*-specific proteins in serum-derived exosomes that differentiated individuals with active and latent TB infection (98, 99). These small-scale studies remain to be verified but suggest that further examination of the biomarker potential of extracellular vesicles is warranted.

microRNA

miRNA are a subset of small RNA (~22 nucleotides long) which are non-coding post transcriptional regulators. Originally considered to be genetic junk, along with other non-coding parts of the genome, miRNAs were first discovered in *Caenorhabditis elegans* and are now known to be master regulators of gene expression and protein translation (100). Many of these miRNAs are highly conserved (101) and play key roles in regulating mRNAs that control complex host signalling networks, as well as immune function. miRNA control the stability (i.e. degradation), translation, and suppression of specific mRNAs in order to regulate a large network of genes and proteins. They have also been indicated in various diseases and as possible drug therapy targets. Their abundance and stability in circulating extracellular vesicles such as exosomes and microparticles have made them potential candidates as disease biomarkers (102-105). Although reports into the role of miRNA in mycobacterial infections, relative to other major diseases, are sparse, their demonstrated differential expression has elevated them to the forefront of mycobacterial research in the last few years. It is currently estimated that over 60% of genes are directly regulated by miRNAs (106), exemplifying the importance of the previously disregarded non-coding aspect of the genome, particularly in regard to biomarker discovery.

There are several mechanisms through which miRNA can exert their “gene silencing” effect, with the degree of miRNA-mRNA complementarity the primary determinant. In general, a high complementarity and perfect to near perfect binding will result in mRNA cleavage, while mismatches

in the miRNA-mRNA complex will reduce protein synthesis through translational repression, a more common phenomenon in animal miRNAs (107, 108).

miRNA biomarkers have been successful in the diagnosis and prediction of outcomes in cancer (109-111) and multiple studies have indicated that miRNA signatures have the potential to distinguish active TB patients from healthy controls and latent TB (112-114). One of the major obstacles to miRNA biomarker investigations is the lack of consistency and established scientific practices, as well as the lack of standardisation across experiments. Variance in case classification, source of biological samples, and study size can affect reproducibility of results making comparison across studies difficult. Variability in miRNA expression due to tissue specificity and miRNA origin, i.e. circulating or exosomal, must also be considered when investigating potential miRNA biomarkers. Further, studies have also indicated that environmental or ethnic differences may also influence miRNA expression (115-117). Analysis of differentially expressed miRNAs in TB has yielded multiple potential biomarker sets yet a rigorous definable signature remains to be confirmed. A large number of miRNAs have been reported to be modulated during TB including the potential biomarkers miR-378, miR-483-5p, miR-22, miR-29c which are upregulated, and miR-101 and miR-320b which are downregulated (118, 119). These miRNAs have been suggested as biomarkers of specific TB disease states, with sensitivity and specificity of 95.0% and 91.8% respectively (119). Similar studies have also suggested that the miRNAs miR-22, miR-25, miR-365, miR-590-5p and miR-885-5p may also be useful in diagnosing TB (118-122). The promising biomarker combinations from human TB research suggests that markers for diseases such as paratuberculosis and bTB may yet be uncovered, and that discovering signatures of resilience to infection are highly plausible.

Several recent studies have focused on miRNA as biomarkers in paratuberculosis and bTB (123-128); however the relatively minor research effort into veterinary diseases compared to TB or similar human diseases has meant that the majority of these studies are still exploratory and further research is required to produce a true diagnostic signature. Potential bovine miRNAs which

may be key biomarkers include immune and inflammatory related miRNAs such as miR-19b, miR-196b, and miR-146, which are modulated during infection and linked to bTB, TB and Crohn's disease (124, 129-135). Although no definitive biomarkers have been elucidated, strong evidence for their modulation following MAP infection indicates that they may be significant candidates for diagnostic markers.

miRNA regulation in mycobacterial infections

Several key miRNAs have been identified in mycobacterial infections, and the similarity in host responses and pathogenesis between mycobacterial species allows for some extrapolation to paratuberculosis and bTB. One of the miRNAs first identified in host immune responses to mycobacteria, miR-146, targets mRNA of TNF receptor associated factor 6 (TRAF6) and IL-1 receptor associated kinase 1 (IRAK1) (136, 137). Acting on TRAF6, miR-146 dampens iNOS and therefore nitric oxide production, an important host microbicidal response (138), while IRAK1 is a key receptor-associated molecule involved in activation of NF-κB transcription (139). Through targeting these molecules, which are essentially downstream signals from TLR cascades, miR-146 can control TLR and cytokine signalling through a negative feedback loop, fundamentally altering the immune response, and decreasing pro-inflammatory effects (Figure 4.).

Another major miRNA modulated by mycobacterial pathogens is miR-142-3p. This miRNA targets an mRNA that negatively regulates a key cell surface signal transducer involved in actin-based cellular motility and assembly of the phagosome for internalised pathogens. miR-142-3p is overexpressed during the early stages of mycobacterial infection and therefore impairs phagocytosis of bacteria (140). miR-142-3p is also a major regulator of pro-inflammatory cytokines, decreasing production and expression of molecules such as TNFα and IL-6, also acting on IRAK1 and the TLR/NF-κB pathway (141).

miR-155 inhibits autophagy and antimicrobial immune effects through ESAT6 inducing expression, preventing immune modulators Cox-2 and IL-6 induction, as well as decreasing Bach1 and SHIP1 (involved in mycobacterial survival and dormancy, as well as production of Reactive Oxygen Intermediates) (142). Nitric oxide production is also limited by increased miR-155 expression in *M. marinum* infections, enhancing survival of pathogenic bacteria (143). As with many miRNAs, miR-155 has multiple functions including modulating the innate TLR response through acting on a number of genes. SOCS1, TAB2 (TLR adaptor molecule) and a DC-specific adhesion molecule are all decreased following overexpression of miR-155, impacting the pathogen binding capability of dendritic cells and possibly contributing to the establishment of disease (144, 145).

miRNAs targeting host cell apoptosis are also modulated by virulent mycobacteria, with miR29a and let-7e upregulated, in turn decreasing caspase 7 and 3 activity respectively (146). As caspase 3 and 7 are both executioner caspases which induce morphological changes for induction of apoptosis, their decreased expression in mycobacterial infections further aids the pathogen in intracellular survival and evasion of immune responses. miR-29 also has a role in decreasing early stage Th1 responses through targeting IFN-γ, with differential expression following infection with both *M. bovis* BCG and *Listeria monocytogenes* (147). miR-582-5p which regulates Forkhead box protein O1 (FOXO1) is upregulated in TB, inhibiting apoptosis by decreasing FOXO1 (148). miR-155 has been implicated as a regulator promoting apoptosis via the TLR2 and PI3K-APT pathways. Pathogenic mycobacteria are able to upregulate miR-155 after activation of TLR2 signalling, and, through a series of cascades and cross-talk between pathways such as MAPK and PKCδ, induce apoptosis by activating caspase 3 and translocating mitochondrial cytochrome *c* (149). miR-21 is also a significant miRNA in apoptosis as it acts on IL-12p35 (IL-12A protein) to decrease IL-12 and therefore activation of Th1 and NK cells. This miRNA also functions to activate apoptosis by targeting Bcl-2, thus further modulating early Th1 responses following *M. bovis* exposure (150).

408 miRNA are also carried within EVs, while exosomal miRNA may be a key regulator of host
409 gene expression and immune defences in mycobacterial infections. Exosomal miR-21 and -29a for
410 example, act as ligands for TLR signalling, suggesting several functional roles and possible roles in
411 paratuberculosis and bTB pathogenesis (151).

412 While these miRNAs clearly play a role in mycobacterial infection, they are only a small
413 number of differentially regulated miRNAs observed in mycobacterial infections and the current
414 understanding of the mycobacteria-miRNA relationships are summarised in Table 4 and Figure 5. It is
415 clear that the regulation, and either over or under expression of these miRNAs, is altered during
416 infection, and their effects are often related to critical events in mycobacterial pathogenesis. The
417 interconnected nature of miRNA, mRNA, and cell signalling pathways are complex. Although current
418 research efforts into the specific functions and modes of action of miRNAs are producing promising
419 results much of the current research focuses on TB; greater investigation into miRNAs and their
420 profiles in bTB and MAP is warranted.

421

422 ## Future directions

423 It is evident, from the nature of mycobacterial diseases, their global distribution and the spread of
424 animal pathogens into the human sphere, that new management strategies are needed to control
425 diseases like paratuberculosis and bTB to ensure subclinically infected animals do not enter the food
426 chain. Directing the focus of production towards identifying animals that are resilient to these
427 diseases may be a means to reducing the economic impact and welfare implications of subclinical
428 infection. Biomarkers are at the forefront here, not only for diagnosis of mycobacterial infections,
429 but also for the differentiation of clinical and subclinical states and identifying resilient animals. In
430 addition, this type of research will undoubtedly provide the ability to characterise immune
431 protection in mycobacterial diseases of animals, which can then be utilised to develop better

vaccines with potential for providing sterile immunity. However, this requires well-designed controlled experimental trials where resilience to disease can be identified accurately. With recent efforts globally to limit the use of antimicrobials in both humans and animals, vaccines can provide advantageous control strategies (152).

The inability to adequately compare current biomarker studies hampers progress. Ideally complete expression patterns of immunologic, proteomic and transcriptomic markers during the course of infection should be studied *in vivo*. The generation of a complete data set would allow for key molecules to be prioritised and a possible combinational signature to be determined. While this would be a large and costly undertaking, the investigation of each of the separate biomarker candidates (e.g. cytokines/chemokines, proteins, genes) from early subclinical to late clinical infection would still provide invaluable information as to the applicability of markers for diagnosis and the host response to mycobacteria. Archived sample biobanks may be integral in these future research efforts, abrogating the cost of establishing *in vivo* infection models and providing multiple sample types i.e blood products and tissue samples, as well as defined infection outcomes and the ability to profile a vast array of biomarker candidates from the same individual over multiple time points. These would also allow the validation of any potential markers across not only different animal species, but also different breeds, which may have differing responses to infection (153). A complete picture of host responses to infection could be obtained through the combination of a variety of 'omics' technologies including transcriptomics, proteomics and metabolomics.

Biomarkers for resilience to mycobacterial infection are a promising resource for better control of for both paratuberculosis and bTB. In our estimation, miRNA are the frontrunners for discovering biomarker signature of resilience. Not only are they ideal biomolecules because of their stability in the circulation and under storage conditions, but additionally miRNA can be isolated from a range of minimally invasive biological sources such as plasma, serum or saliva. They are master regulators of gene expression and mediate many biological and metabolic processes, thus are

upstream of the transcriptomic, proteomic and metabolomic effects. Changes in their expression and patterns of regulation are likely indicators not only of infection, but also of the disease phenotype and/or resilience to mycobacterial disease. One drawback could be their inability to be pathogen-specific; to overcome this limitation, there may be a diagnostic role for a combined pathogen-specific cytokine or chemokine (e.g. IFN-γ) response and miRNA signature to identify resilient animals. With rapid advancements of biomarker discovery platforms such as next-generation sequencing and array technologies we envisage the capacity to develop of robust signatures for significant global diseases.

Table 1. Cytokine and chemokine responses to mycobacterial infections based on transcriptomic and proteomic data

SPECIES	HOSTS	SAMPLE	IMMUNOLOGICAL MARKER	REFERENCES
M. avium subsp. *paratuberculosis*	Sheep, cattle, goats, camelids, deer	Bovine/ovine intestinal tissue	↑: TRAF-1, IL-8, IFN-γ, TNF-α, IL-10, IL-12, TGF-β, IL-1α, IL-1β, IL-6 ↓: IL-18	(54, 154)
		Bovine plasma + MDMs	↑: IFN-γ, Osteopontin ↓: IL-4 ↑↓: IL-17	(56)
		THP-1 cell line	↑: TNF-α, IL-1β, IL-10	(155)
		Bovine PBMCs + intestinal tissue + lymph node	↑: IFN-γ, IL-1α, IL-1β, IL-5, IL-6, IL-8, IL-2, IL-10, IL-4, IL-2R ↓: IL-16, IL-18 ↑↓: TGF-β	(55, 156)
		Murine spleen/liver/ileum	↑: IFN-γ, TNF-α, IL-4	(157)
		Caprine PBMCs	↑: NOS2, IL-21, C2, C3, IL-34, IL-12A, TLR4, TNF, ↓: IL-17F, IL-9, IL-9R2, IL-36β, IGF1, IL-18, IL-9, IL-5, IL-13, IL-11, Granulysin, IFN-γ	(158)
		Whole blood (bovine)	↓: TNF-α, RANTES, MCP-1	(67)
M. bovis	Cattle, possums, badgers, buffalo	Murine spleen/lung + bovine PBMCs	↑: IFN-γ, IL-22, CXCL9, IL-17a, IP-10, Granzyme B, IL-17Re, Granzyme A	(57)
		Multinucleated giant cells	↑: TNF-α, IL-17A, TGF-β, IL-10, IFN-γ	(159)
		Bovine PBMCs	↑: IFN-γ, TNF-α, iNOS, IL-4 ↓: IL-10	(160)
		Bovine lymph node	↑: IFN-γ, TNF-α, TGF-β, IL-17A ↓: IL-4, IL-6, IL-10, IL-22	(161-163)
M. marinum	Fish, frogs, humans (NTM)	Goldfish spleen/kidney + leukocytes	↑: ROI, NO, IL-1β, IFNGR, TNFR ↑↓: SOCS3, TGF-β, IL-10	(164)
		Murine mast cells + HMC-1	↑: COX-2, TNF-α, NOD2	(165)
		Adult zebrafish (homogenised tissue)	↑: MMP13, TNF-α, IFN-γ, IL-1β	(166)
		Human Mφ culture supernatant	↑: IL-12p40, IL-6, TNF-α, ↓: IL-1β	(167)
		Kidney Mφ (goldfish)	↑: NRAMP, IL-10, TGF-β1, SOCS3, TNF-α, IL-1β1, IFN-γ, CXCL8, IFN-γrel, IDO, CCL1 ↓: ROI	(168)
M. hominissuis	Pigs, humans,	Human PBMCs	↑: IL-17 ↓: IL-12p70	(169)
M. avium	Poultry, humans	Human PBMCs + alveolar Mφ	↑: IL-10, IL-17, TNF-α, IFN-γ ↓: IFN-γ, IL-12, IL-12p70	(169, 170)
M. leprae	Humans, armadillos, primates	Human PBMCs	↑: IL-4, IL-6, IL-8, TNF-α, TGF-β	(171)
		Human Schwann cells	↑: TLR2, TLR4, MyD88, Irak4, IL-18, CCL2, CCL7, CCL9, CSF-1, Mif, CXCL1 ↓: TLR1, TLR6	(172)
M. smegmatis	Soil – rarely found in animals or humans	RAW 264.7 cell line	↑: TNF-α, IL-6, MCP-1	(173)
		Human PB Mφ	↑: IL-1, IL-6. TNF-α, GM-CSF-	(174)

SPECIES	HOSTS	SAMPLE	GENE	REFERENCES

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Table 2. Differentially expressed genes in mycobacterial infections of animals

M. avium subsp. *paratuberculosis*	Sheep, cattle, goats, camelids, deer	Whole blood (bovine)	↑: *KLRB1, MPO, LTF, SERPINE1, S100A8/9, TFRC, GBP6, PIGR, IL-10, CXCR3, CD14, ELANE, CHI3L1, HP, HGF, MMP9, DEFB1, DEFB10, TIMP1, PIP5K1C, IRF5, IRF7, CORO1A* ↓: *IL17F, IL17F, IL22, IL26, HMGB1, IRF4*	(74, 75, 175)
		THP-1 cell line	↑: *CD14, CD68, S100A8/9, ELANE, LTF, HP, CCL4, CCL5, CXCL9, CXCL10* ↓: *ELANE, IGF1, TCF7L2, MPO*	(76)
		RAW 264.7 cell line	↑: *ABCA1, APOE, LDLR, RFTN1, HMCGR, IL1A, IL1B, IL6, MCP1, TNFA, INOS, LAMP1, P53, TLR4 PLIN2, SREBF1, RAB7* ↑↓: *TFRC, CXCR31, CCNE2, COX62A, GDF15, YPEL3, AQP9, SLC40A1, TMEM154, CD74, AATK, RRAS, GADD45α, YPEL5, HEBP1, ENO2, MACROD1, IRF7, NFKBIζ, LCN2*	(176, 177)
		Bovine monocytes + WBCs + PBMCs	↑: *TGFB, TSP1, BCL2L1, TGF, IL6, MMP12, MT1A/B/E/F/H/I, 17A-HYDROXYLASE, CD40L, CRF, CRFR1, EP2, FSG-R, IL1, IL10, IL12, IL2, IL4, IL5, IFNG, MMP1, MMP3, MMP7, MMP9, MMP15, MMP 16, MMP19, MMP23, PAI1/2, SCC, SPARC, TGFB, TIMP1, TIMP2, TIMP2 V3* ↓: *SFK, ADRB, cAMPPK, VTAP, TNFB, DQB, IA6, MAPK2K5, MEK5B, CD38, GIMAP6, SCD-1, 24DHCR, LDLR*	(178-180)
M. bovis	Cattle, possums, badgers, buffalo	Lymph nodes + tonsils + spleen (wild boar)	↑: *VDR, ANX, LAP, VCAM, CXCR4, MHC-I SLA-31, B2M, MHC-II SLA-DRA, C3, C7, HSPGP96, LYZS, ARG, OPN, CUL, ARP3, MUT, DEFB129, BAP29, CD8A* ↑: *LGALS1, C1QB, CD74, SLA*	(181, 182)
		Bovine PBMCs + MDMs	↑: *PPP2R5B, ZDHHC19, 28S, GPR98, PDGFA/B, ECGF1, MHCR1, AXL, CD84, CCL15, NFATC4, TLR2, CD80, NFKB1, IL8, CXCL6, ADORA3* ↓: *PRKCB1, PRKCA, AKT1/2, EEF2, EEF1G, GATA4, IER5, CSF2, CD14, CCL1, CHUK, NFKB1, TBK1, MIF, CCR7, BOLA, ADAM17, CXCR3, PHB2, STK17B, MCL1, CCL1, IL8, TLR2, TLR4, BCL2, NCOR1, UCP2, UNC84B, GAN, SFPQ, NRM, FGFR1,*	(80, 183)
		Whole blood (bovine)	↑: *CD83, CTLA4, IL1A, IL8, STAT1, TLR4,* ↓: *CASP1, DEFB10, IFNGR2, IL15, KIR3DS1, MYD88, STAT2, TLR3, TREM1, TYROBP*	(184)
M. marinum	Fish, frogs, humans (NTM)	Muscle wound tissue + homogenised zebrafish	↑: *ATF3, BCL3, CEBPB/D, ELF3, IRF1B, IRF3, FOSL2, JUNBA/B, NFKB, IL1B, TNF, CXCL8A/B, MMP9/13A, TIMP2B, C3/7/8/9, IRG1, SAA, STEAP4, HAMP, DRAM1, IRAK1, SOCS3, NCF, NOX, CYB, ILIB, TNFAIP2/3/6* ↓: *CKMA/B, MYLPFA, MYLZ3, MYL10, ACTA1B, MYOZ1A, MYOM1A, MB, MYBPC2A, MURCA, MYOM2, MYL1, MYOZ3A* ↑↓: *APOA, APOE, APOB, FOSL1A, FOSAB*	(185, 186)
M. hominissuis	Pigs, humans,	Human MDMs	↑: *INHBA, CCL1/3/4/5/18/20, ILI, VEGFC, MMP1/3/10, SLAMF1, CCR7, TNFAIP6, TNIP3, IL7R, PROCR, PDGFB, CSF2, TNF, IL8, IL3RA, BMP6, MSC, TM4SF1, TNFRSF9/19, MRC1, LAMB3, CHST2, ETS2, PTGS2, IL10, SOCS3, SERPINB2, SERPINE1, TIMPI, BTG1, SOD2, CD14, PLAUR* ↓: *STMN1, LTA4H, CD36*	(146)
M. avium	Poultry, humans	U937 cell line	↑: *ERBB3, EPHA3, PTPN7, LAT, CSF1, NFKB, JUN, SPI1, ARHGDIA, GNB1, GNB2L1, FGF11, ITGA5, ITGAL, ICAM1, IEX1L, CASP10, RPS19, TNFA, RANTES, MIP2, ILIB, IL8, IL2RA/G, TNFRSF1B, CDKN1A, TIMP1, MMP9/11, CAPN4, PI, AZU1, MT1H, DTR* ↓: *ID2, SPN, BCL2L1, TMSB4X, AP2M1, CTSD*	(187)
M. leprae	Humans, armadillos, primates	FFPE leprosy lesions	↑: *NOD2, TNFSF15, RIPK, CCDC122, HLA-DR, C13ORF31, LRRK2*	(188)
		Whole blood (human)	↑: *VEGF, GNLY, GZMA/B, PRF1* ↓: *IGF, KIF1B, LRRK2*	(189)
M. smegmatis	Soil – rarely found in animals or humans	U937 cell line	↑: *CDKN1A, ERBB3, BRF1, NSEP1, JUN, GNB1, FGF11, GRN, PGF, NDUFB7, ICAM1, IEX-L1, LIF, RANTES, MIP2, ILIB, TNF, IL8, SPP1, IL2RG, MMP1/9, HSPA1A, FTH1, BTG1* ↓: *IQGAP1, CRHR1*	(187)

SPECIES	HOSTS	SAMPLE	PROTEIN/PEPTIDE	REFERENCES
M. avium subsp. *paratuberculosis*	Sheep, cattle, goats, camelids,	Bovine serum	↑: VDBP, thransthyretin, RBP, alpha-2 glycoprotein, SERPINA3, cathelicidin, VDBP precursor, leucine-rich alpha-2-glycoprotein	(82)

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Table 3. Dysregulated protein responses to mycobacterial infections of animals

SPECIES	HOSTS	SAMPLE	miRNAs	REFERENCES
M. avium subsp. *paratuberculosis*	Sheep, cattle, deer goats, camelids,	Bovine whole blood	↑↓: Fetuin-B, serine protease inhibitor, alpha-1B-glycoprotein, alpha-1 acid glycoprotein ↓: miR-19b, miR-19b-2, miR-1271, miR-100, miR-301a, miR-32a	(130)
		Bovine plasma	↑: Transferrin, gelsolin α/β, actin binding protein, C1r, C3, AOC3, thrombin ↓: COAFXIII, FCG	(84)
		Camelid serum	↑: Hp, serum amyloid A, Fb	(190)
		-	FbpA/B, FbpC2, PirG, Wag31, MetC, PepA, Csp, modD, thioredoxin, thiol peroxidase, FadB4, FabG5_2, FabG3_2, AhpC, Hsp7-, Hsp65/K, superoxide dismutase, FixA, pstA, EchA20/8_1, DesA2, MoaA3	(191) (Review)
M. bovis	Cattle, possums, badgers, buffalo	Bovine serum	↑: Alpha-1 antiproteinase, fetuin, VDBP, alpha-1 acid glycoprotein, alpha-2 glycoprotein 1, alpha-1-B glycoprotein, RBP, Pks5 ↓: SERPINA3	(81, 82)
		Buffy coat (bovine)	↑: TLR2/4/9, MHC1, Syngap1, Alox5, Adar, Mpo, tyrosine-protein kinase, Pxk, MHCII, ↓: C8α/β, TINAGL1, Drosha, Ifnκ, PIK3C2B, Tyk2, P2x, IL1RL2, oligoadenylate synthase, protein kinase C, beta-1,4-galactosyltransferase 1, CXCL2, Lif, thrombspondin-1, AP-3, azacytidine-indiced protein, CCL20 ↑↓: 8B, ADAM15, Rnf19B, PLAA	(192)
		THP-1 cell line	↑: Sod2, Krt99, CCL20, ICAM1, Ncf1, Tnnt1, Vps26A, Apoe, Rbm17, Agtrap, REP15, Cmtm6, Pklr, Yars2, CCDC124/51/93, Dpysl4, Acaa1, Mthfd2, Ckap4, Derl1, Ndrg1, LAMTOR2, TBC1D9B, Rnf2 ↓: Tma7, Mtpn, Tmsb10, Tmsb4X	(193)
M. marinum	Fish, frogs, humans (NTM)	Murine BMDMs, RAW 264.7 cell line & THP-1 cell line	↑↓: ESAT-6, CFP-10, LC3, MMP13, Arp2/3, WASP, N-WASP	(166, 194, 195)
M. hominissuis	Pigs, humans,	BEAS-2B cell line	↑: Snd1, NADPH dehydrogenase, Ddx6, Cbr1, Importin, Exportin-5, Cndp2, Dynamin-1-like protein, HNRPK/L, Pafah1B3, GCP60, Ubap2L, glutathione synthetase, PPP2A, calnexin, Banf1, lactoferroxin-C, MBP-1	(196, 197)
M. avium	Poultry, humans	U937 cell line	↑↓: CAM1/2/3, PPP3R1, Dffa, Bub3, Smc1A, CDK1, CycB, HDAC2, TUBA1B, ItgB2, UBA1, ACTB, H1.4, PP1, PP2A, ITGA	(198)
M. leprae	Humans, armadillos, primates	-	↑↓: PGL1, ErbB2, α-DG, laminin-2, MMP1/2/9, IDO, VDR, SMAD, VD, SLC11A1	(199, 200) (review)
M. smegmatis	Soil – rarely found in animals or humans	Murine BMDMS & BMDDs	↑↓: Calmodlin, cAMP, CREB, caspase-8, caspase-3	(201)

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Table 4. miRNA responses to mycobacterial infections of animals

	deer	Bovine intestinal tissue	↑: miR-146b, miR-1247, miR-196b, miR-184 miR-202 ↓: miR-137, miR-105a, miR-433, miR-133b	(124)
		Murine BMDMs	↓: miR-27a-3p	(202)
M. bovis	Cattle, possums, badgers, buffalo	Bovine alveolar Mφ	↑: miR-146b, miR-146a, miR-147, miR-29c, miR-22-3p, miR-21-3p, miR-142-5p, miR-210, miR-32, miR-125a, miR-155, miR-99b, miR-27a-5p, miR-149-5p, miR-28, miR-15a, miR-23a, miR-29a, miR-30b-5p, miR-151-5p ↓: miR-92a, miR-34a, let-7a/b/c/d/e/f, miR-6529, miR-107, miR-744, miR-328, miR-423-3p/5p, miR-345-3p, miR-128, miR-874, miR-378b, miR-296	(126)
		HEK293T, EL4 cell lines + human MPMs	↓: miR-29a	(147)
		Human MDMs (BCG)	↑: miR-135b, miR-296-5p, miR-645 ↓: miR-629	(203)
M. marinum	Fish, frogs, humans (NTM)	RAW 264.7, THP-1, HEK293T cell lines + MPMs	↑: miR-155	(143)
		Adult zebrafish (homogenised tissue)	↑: Let-7a/c/d, miR-142b, miR-146a-3p/5p, miR-146b-3p/5p, miR-15c, miR-16b, miR-181a, miR-181b, miR-20b, miR-21-3p/-5p, miR-219, miR-223-3p/5p, miR-23b, miR-26a, miR-29a, miR-29b, miR-430a/i, miR-457b, miR-462, miR-728-3p/5p, miR-731-3p/5p, miR-732 ↓: miR-10d, miR-25, miR-30b/c, miR-128, miR-150, miR-181c, miR-184, miR-204, miR-216a/b, miR-217, miR-365, miR-430b, miR-454b, miR-461, miR-489, miR-724, miR-727, miR-730	(204)
M. hominissuis	Pigs, humans,	Human MDMs	↑: miR-155. miR-146a, miR-146b-5p, miR-886-5p ↓: miR-20a, miR-191, miR-378, miR-30c, miR-423-5p. miR-374a, miR-185, miR-768-5p, miR-18 ↑↓: let-7e/i, miR-146b-5p, miR-29a, miR-193a-5p, miR- 483	(146)
M. avium	Poultry, humans	Human MDMs	↑↓: miR-29a, let-7e, miR-146a	(146)
M. leprae	Humans, armadillos, primates	Skin biopsy	↑: miR-21, miR-24, miR-146a, miR-451, miR-30a/b/e, miR-22, miR-181b, miR-34a, miR-93, miR-422a, miR-29c	(205)
		Skin biopsy	↑: miR-142-3p/5p, miR-146b-5p, miR-342-3p/5p, miR-361-3p, miR-3653, miR-484, miR-155, miR-146, miR-21, miR-150. miR-181 ↓: miR-1290, miR-429, miR-141, miR-205, miR-193b, miR-200c, miR-224	(206)
M. smegmatis	Soil – rarely found in animals or humans	Human MDMs & J774A.1	↑: miR-125b, miR-142-3p ↓: miR-155	(207, 208)

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Figure Legends:

Figure 1. Immunological markers predictive or associated with stages of mycobacterial infection. Following exposure to mycobacterial pathogens, hosts may have either a successful immune response to eliminate the bacteria before an infection is established, or may progress along the spectrum of disease. When the pathogen remains in the host system and is able to persist, the infection remains latent/subclinical. At this stage in latent bTB, the IFN-γ and proinflammatory response is also elevated, and coupled with a decrease in anti-inflammatory IL-10. In paratuberculosis, during the subclinical infection stage, there is an increase in a number of proinflammatory cytokines. From here, the animal may successfully control the infection and eliminate the bacteria (termed 'Resilience'), or progress to clinical disease. An early, elevated IFN-γ and antibody response is observed in infected sheep that progress down a pathway of Resilience to disease. During clinical disease, the response is primarily anti-inflammatory, with a decrease in key proinflammatory cytokines. A similar response is observed in active bTB, where the immune responses favours anti-inflammatory cytokines such as IL-10 and TGF-β. Elevated IP-10 levels may be predictive of animals that will develop active bTB.

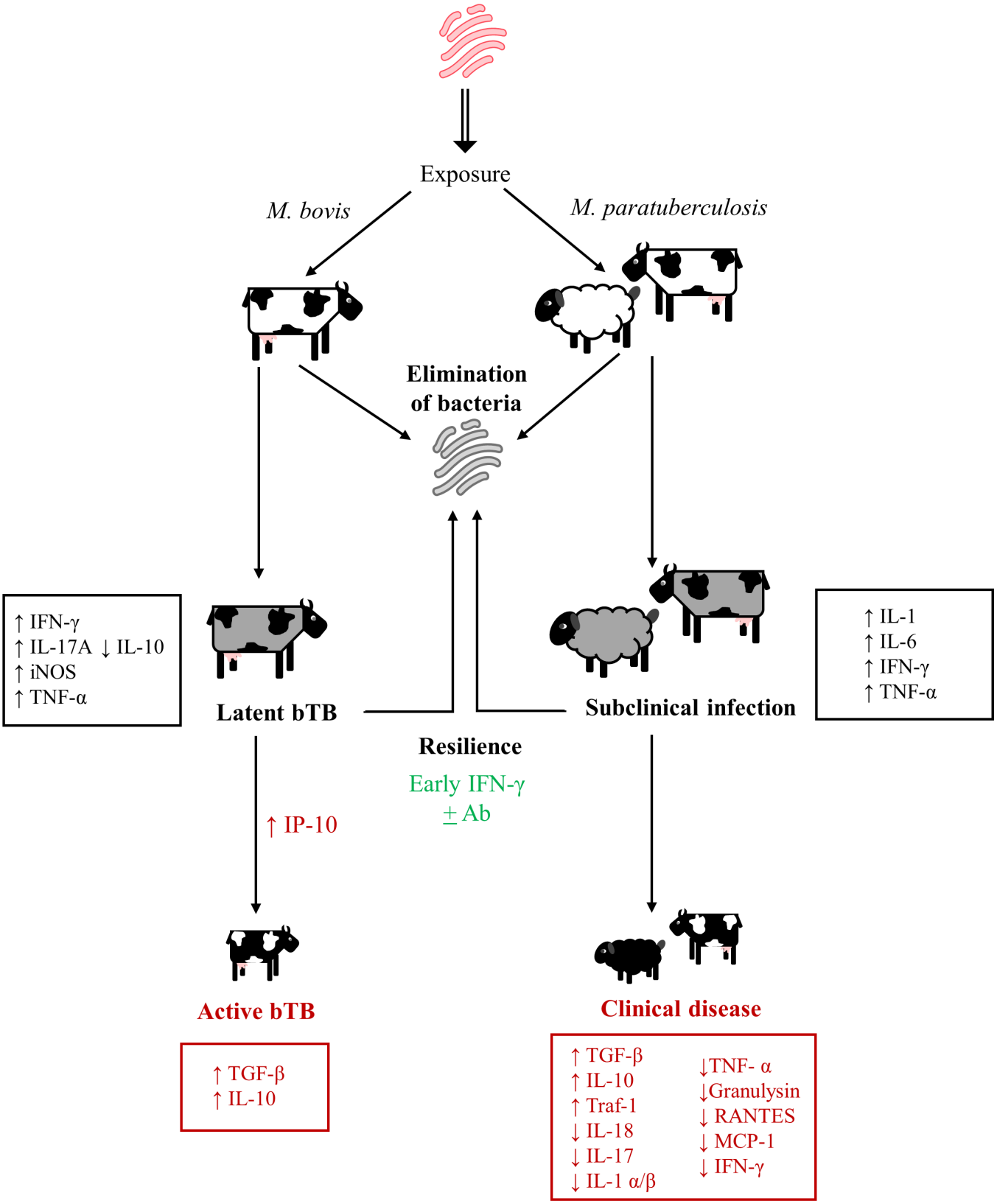
Figure 2. Host biomarker responses to mycobacterial infection. Potential biomarkers for mycobacterial infection play many different roles in the host response. Some commonly measured biomarkers and the likelihood of either a successful host response or successful modulation of the response by the pathogen are shown here. Vitamin-D is a key antimicrobial agent involved in mycobacterial infections. Host upregulation of the Vitamin D receptor (VDR) and the subsequent binding of Vitamin D (D25/calcitriol) triggers nuclear translocation and specific cellular responses. A resulting increase in genes such as *Defb1/10* and the production of antimicrobial defensins reduce bacterial burden and facilitate mycobacterial killing. An opposing response favouring mycobacterial persistence is associated with an increase in IL-10 and a subsequent upregulation of STAT3 transcription. Acting through MARCH1, STAT3 is able to reduce MHCII expression and therefore reduces further antigen presentation. Concurrently, increased levels of STAT3 block the release of chemoattractant signals from IL-12 to prevent an influx of immune cells.

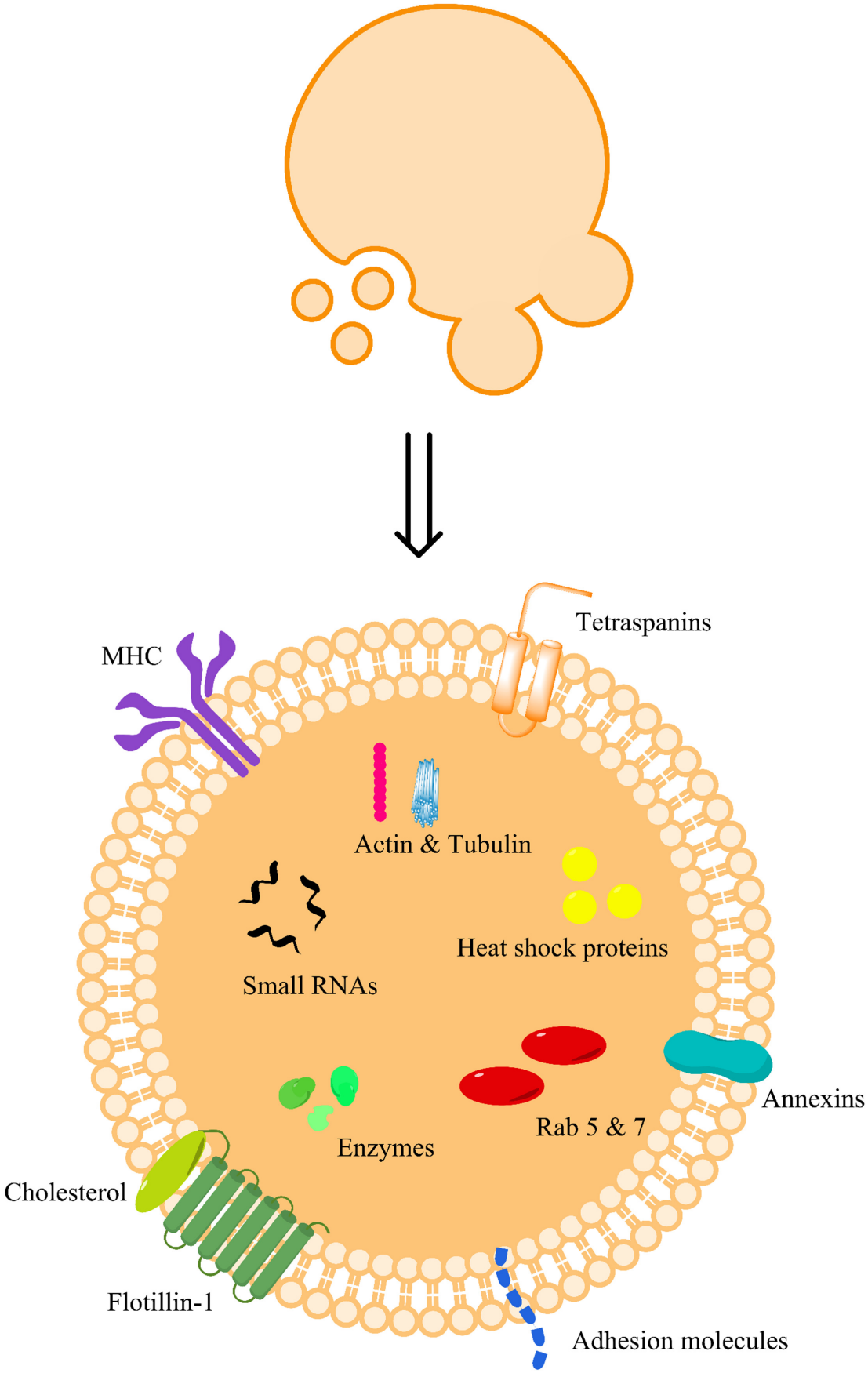
Figure 3. General extracellular vesicle structure. A phospholipid bilayer membrane surrounds the vesicle and contains several key molecules: annexins assist in transport and membrane fusion, lipid rafts consisting of flotillin-1, cholesterol etc. aid in internalisation,

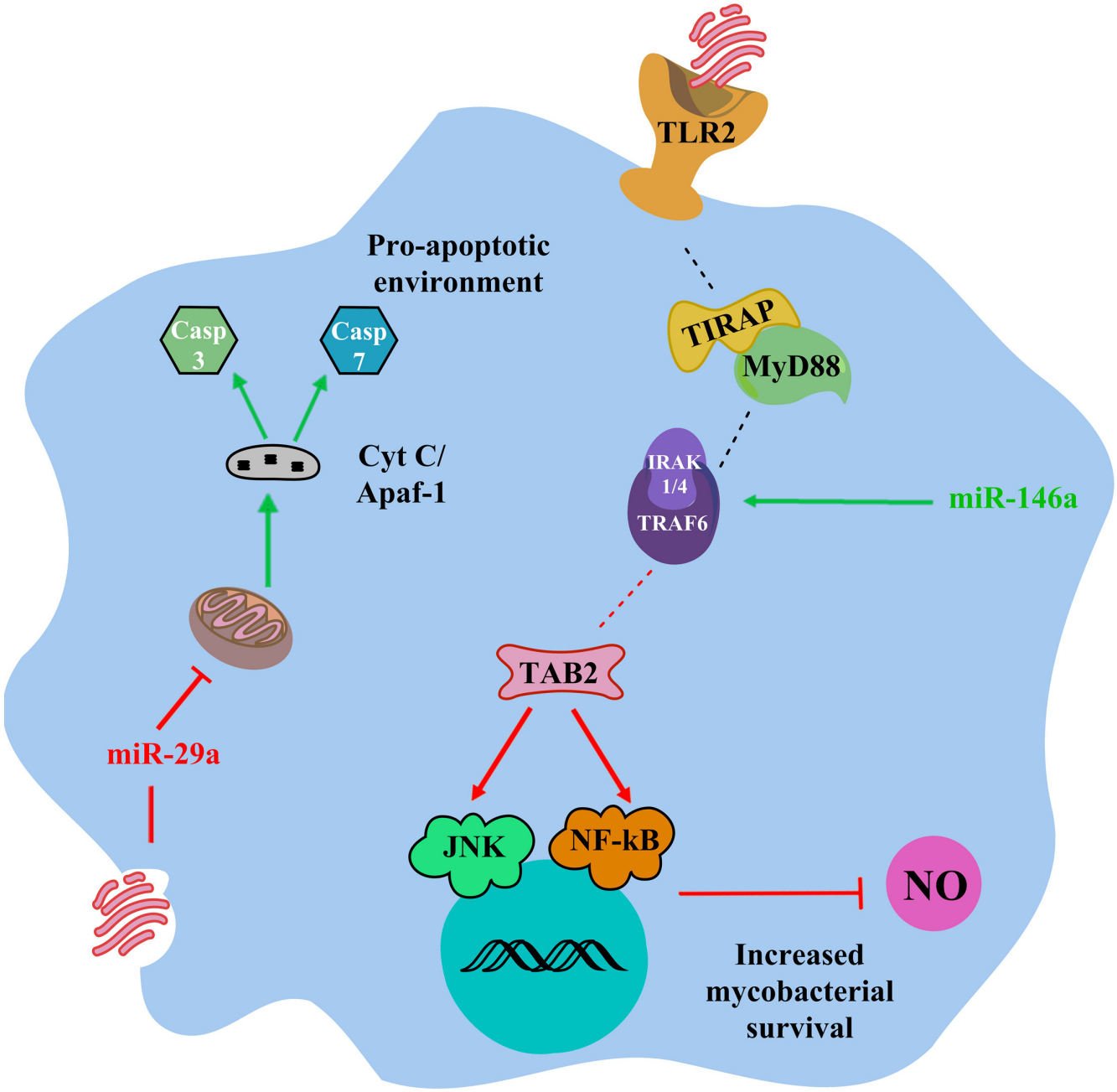
MHC class I and II enables peptide binding, adhesion molecules such as β2 integrin and ICAM-1, and tetraspanins such as CD63 and CD81 are for cell recognition. The internal compartment also contains a range of important components including miRNA, Rabs for exosome docking, HSPs to aid in MHC peptide binding, and cytoskeletal proteins.

Figure 4. miRNA responses to mycobacterial infection. Conflicting miRNA responses are common in bacterial infections, resulting in either pro- or anti-survival conditions, with an example of each given here. Upon encountering mycobacteria, miR-29a can be either up or downregulated. When miR-29a is decreased, its effect on mitochondrial membrane potential is lessened, allowing for the release of cytochrome c and eventual activation of caspases which result in cell death and possible bacterial clearance. In contrast, recognition of mycobacteria by TLR2 and MyD88/TIRAP results in an increase in miR-146a, which directly targets and reduces TRAF6. This reduction leads to a decrease in iNOS and NO production, and an overall decline in mycobacterial clearance. The specific miRNA response is dependent on the pathogen and host immune response and may therefore contribute to the disease progression and phenotype.

Figure 5. miRNA responses to mycobacterial infection. Infection and exposure to mycobacteria results in a large-scale miRNA response with changes in different functional and biological pathways. The above miRNAs are some that have been observed as being dysregulated during infection and their function identified. There are likely many other miRNAs that are of importance in mycobacterial infections which fall into these, and other, canonical pathways.







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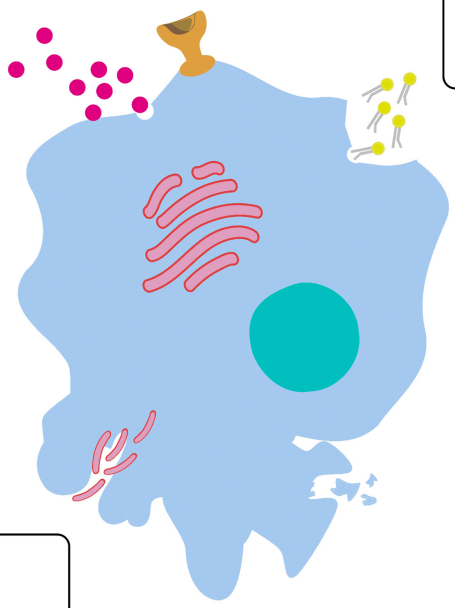

TLR signalling-Cytokine/ Inflammatory Response

Let-7 family
miR-19b
miR-1271
miR-301a
miR-146a/b
miR-105a
miR-433
miR-147
miR-29
miR-142
miR-99b
miR-28
miR-30b
miR-744
miR-181
miR-223
miR-26
miR-150
miR-365
miR-191
miR-451
miR-342
miR-224

Lipid Pathways

miR-378
miR-202
miR-27a-3p
miR-125
miR-107
miR-422

Autophagy

miR-142
miR-125
miR-23
miR-30b
miR-92a
miR-423

Apoptosis

miR-24-1/2
miR- 100
miR-184
miR-133b
miR-27a-3p
miR-202
miR-29
miR-21-3p
miR-149
miR-15a
miR-34a
miR-874
miR-645
miR-16b
miR-26
miR-731
miR-886
miR-25
miR-193
miR-34