Brain Tumour Foundation of Canada – Research Grant - Project Report

"Understanding the impact of tribbles proteins on glioblastoma development"

I. SUMMARY

Malignant gliomas are the most common and deadly brain tumors. Last year, more than 2,600 cases of brain tumors were reported in Canada and the mean survival rate for a patient diagnosed with a glioblastoma multiforme (GBM), the most aggressive glioma, remained slightly over one year. Current standard of care primarily consists of treatment with the agent temozolomide concurrently or following radiotherapy, yet molecular mechanisms that can contribute to temozolomide resistance in GBMs are not well characterized. Improving our knowledge of the metabolic cascades associated with inherent and acquired temozolomide resistance in GBMs is, hence, of uttermost importance and was investigated as part of this research grant. Results collected within the current research program serve as a springboard to (1) develop a combinatorial therapeutic strategy directed towards these new targets and aimed at circumventing temozolomide resistance in GBMs and to (2) identify a signature of metabolites in temozolomide-resistant GBM patients that will pave the way for the development of an early detection test of patients not responding to temozolomide. In addition, it is important to mention that the financial help provided by the Brain Tumour Foundation of Canada was instrumental in assisting a young investigator such as Dr. Morin setting up a strong research environment in Eastern Canada and ensuring that this quickly translated to funding at the national level. This grant undoubtedly contributed to these objectives as key results were generated, conferences were attended, several publications were written and, most importantly, our understanding of the molecular footprint associated with GBMs was greatly improved.

II. BACKGROUND

Glioblastoma multiforme (GBM) is a common and malignant subset of brain tumors that is classified as a grade IV astrocytoma by the World Health Organization (WHO). The deadliest form of glioma, GBMs are associated with a 14.6 months median survival rate, despite the aggressive use in combination of surgery, radiation and chemotherapy. The use of the alkylating agent temozolomide (TMZ) concurrently and after radiotherapy has improved patient survival marginally and has become a common therapeutic option for a sub-group of GBM patients. Unfortunately, TMZ activity is often antagonized by DNA-repair enzymes leading to treatment resistance in GBMs. Alternative therapeutic approaches, as well as a better understanding of the mechanisms underlying TMZ resistance, are thus seriously needed for patients diagnosed with a GBM. These two overarching objectives have been tackled by our research group over the past few years:

(1) Alternative therapeutic approaches - The protein kinase family consists of more than 500 proteins that act as key regulators of cellular signaling. Protein kinases are thought to regulate via phosphorylation an estimated 30% of all human proteins. This makes them a focal point in cellular signal transduction and appealing drug targets. In fact, one of the most common oncogenic events observed in GBMs is the amplification and overexpression of the EGFR kinase, occurring in 40-60% of primary GBMs. Sustained activation of the EGFR cascade leads to hyperactivation of key protein kinases such as PI-3K/Akt and mTOR. Hence, deregulated members of the protein kinases family modulating intracellular growth signaling are potential therapeutic targets for GBMs. Several members were characterized, to different extent, during the course of this grant, including tribbles proteins, and are presented below.

(2) Mechanisms underlying TMZ resistance - In addition to kinase signaling, metabolic alterations are also required for malignant transformation and deregulated cellular metabolism is considered as a crucial hallmark of cancer. Understanding how cancer cells derive energy and necessary building blocks is of fundamental importance for the development of appropriate therapies and diagnostic approaches. High throughput metabolite profiling - metabolomics – can be leveraged to analyze these metabolic differences. Metabolomics is particularly important in the search for biomarkers that can eventually be used for *in vivo* diagnosis and prognosis. TMZ, the therapeutic option of choise for GBMs, is an alkylating agent that adds a methyl group to purine bases of DNA (O6-guanine, N7-guanine and N3-adenine). Mechanisms that can reverse this methylation and contribute to TMZ resistance in GBMs are starting to emerge. Understanding the metabolic cascades associated with inherent and acquired TMZ resistance in GBMs is crucial in order to improve the therapeutic alternatives offered to TMZ-resistant GBM patients. Metabolic profiling associated with TMZ resistance and sensitivity was performed in GBM cells during the tenure of the grant and results are shown below. These profiles set the

stage for the development of diagnostic tool for early detection of TMZ resistance in GBM patients not responding to this agent.

III. RESULTS

(1) A microarray-based approach that measured transcript levels of all human kinases (the kinome) in GBM tumors was performed (Dr. Brian A. Hemmings' laboratory, Friedrich Miescher Institute, Basel, Switzerland). Normal human astrocytes (NHA) and normal brain tissue samples were used as controls. Several kinases transcripts showed increased expression in primary GBM samples. An antibody microarray was also used to identify signaling proteins that were differentially regulated in GBMs. Two kinase candidates, Mnk1 (figure 1) and Mertk, were shown to be particularly important in driving gliomagenesis and findings were reported in the literature (Grzmil et al., 2011, Wang et al., 2013). Besides these kinases, the tribbles proteins Trib1 and Trib2 stood out as other interesting overexpressed candidates underlying GBMs (figure 2). Trib1 protein levels were subsequently measured via immunoblotting in a panel of GBM cells and compared to NHA. A sub-set of GBM cells, including Hs683, LN18 and LN405, were shown to significantly overexpressed Trib1 (figure 3). Trib1 protein levels were also measured in a panel of primary GBM samples by immunohistochemistry. Trib1 levels were shown to be highly expressed in a significant portion of GBM samples while Trib1 levels were negligible in the normal brain samples (figure 4). Interestingly, an inverse relationship between Trib1 protein levels and C/EBPα transcript levels was observed in GBM cell lines (figure 5). This relationship between Trib1 and the C/EBPα transcription factor had been previously reported in myeloid leukemogenesis. These preliminary findings thus suggest a potential involvement of Trib1 and C/EBPα in GBM development. Subsequent work will be aimed at characterizing this interaction further.

(2) A metabolomics-based approach was performed to better characterize the metabolic footprint associated with a panel of GBM cell lines available in Dr. Morin's laboratory before undertaking any pharmacological treatment. Interestingly, this NMR-metabolomics approach demonstrated distinct clustering between the GBM cells analyzed based on their metabolites profiles. Analysis showed that some of the major cancer metabolic markers such as choline, creatinine and lactic acid had significantly variable concentrations in different GBM sub-groups (Figures 6-7, Table 1). As part of this work, it was also discovered that GBM cells U373 and LN229 were sensitive and resistant to TMZ, respectively (Figure 8) and that a distinct metabolic profile existed between these cells (Figure 9). These include glycerol-3-phosphate, a metabolite with potential relevance to drug (imatinib) resistance in leukemia, and isoleucine, an amino acid that can discriminate between different cellular bioenergetics profiles in cancer cells. These results thus allowed us to identify several GBM sub-types based on their metabolic profiles further confirming the heterogeneity of this malignancy. It is important to mention that a TMZ-resistant cell line (U373R) was also developed from a sensitive counterpart (U373) and these models will be leverage to better assess the metabolic footprint of TMZ resistance (Figure 10). This footprint, as well as the development of pharmacological inhibitors that could help overcoming TMZ resistance in GBMs, will be investigated by three students (1 MSc, 2 BSc) in our laboratory during the upcoming year.

IV. FIGURES AND TABLES

Figure 1.

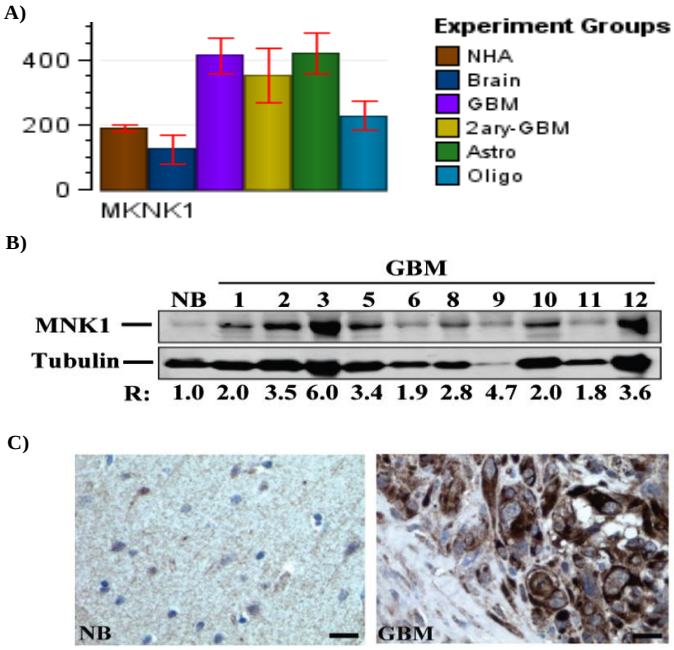


Figure 1. (A) *Mnk1* transcript expression in primary brain tumours. Relative *mnk1* transcript levels were assessed by microarray in glioblastomas (primary and secondary), astrocytomas (astro) and oligodendrogliomas (oligo) primary tumours. Normal human astrocytes (NHA) and normal brain (brain) tissue were used as controls. Histogram shows spot intensity measured on Affymetrix microarrays. (B) Mnk1 protein levels assessed by immunoblotting in a panel of primary GBM tumours. (C) Mnk1 protein levels measured by immunohistochemistry in a GBM tumour sample. (B) and (C) from Grzmil *et al.*, 2011.

Figure 2.

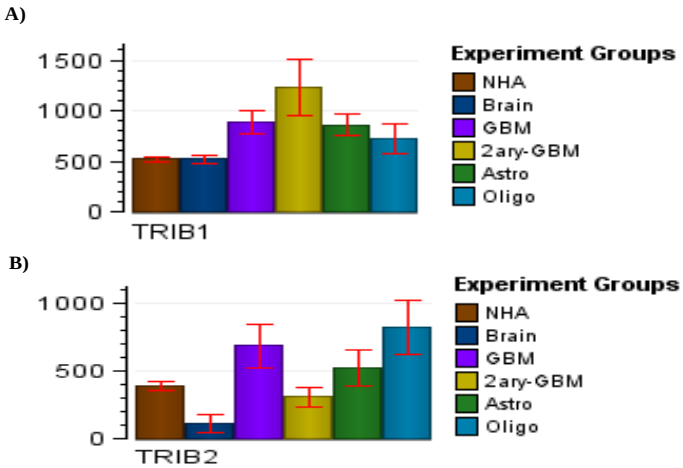


Figure 2. Selected tribbles transcript expression in primary brain tumours. Relative *trib1* (A) and *trib2* (B) transcript levels were assessed by microarray in glioblastomas (primary and secondary), astrocytomas (astro) and oligodendrogliomas (oligo) tumours. Normal human astrocytes (NHA) and normal brain (brain) tissue were used as controls. Histogram shows spot intensity measured on Affymetrix microarrays.

Figure 3.

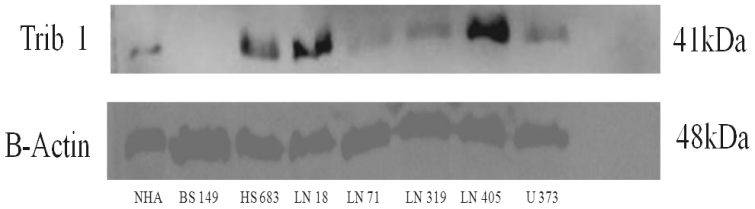


Figure 3. Tribbles transcript and protein levels measured in a panel of glioma cell lines by Western blotting.

Figure 4.

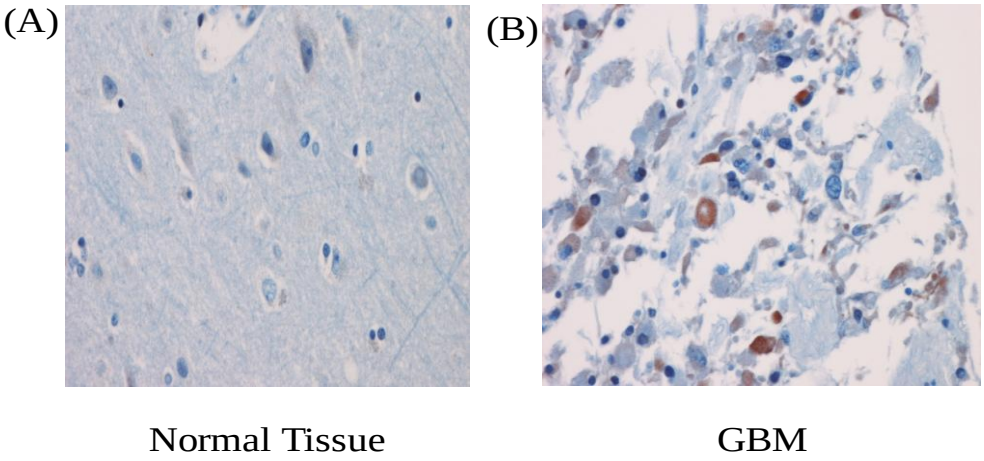


Figure 4. Immunohistochemistry. Normal brain tissue and 45 GBM samples were probed with a Tribbles 1 antibody. Five astrocytomas were also analysed. Panel A: Protein expression of Trib-1 in normal tissue. Pannel B: Protein expression of Trib-1 in GBM samples.

Figure 5.

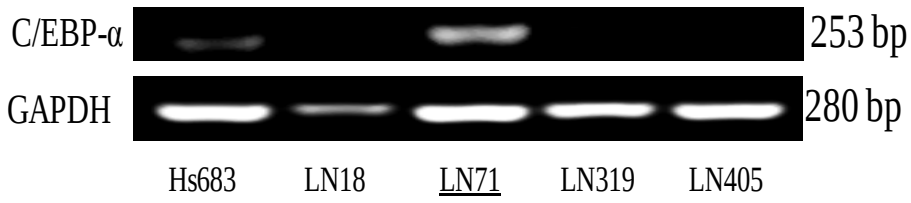
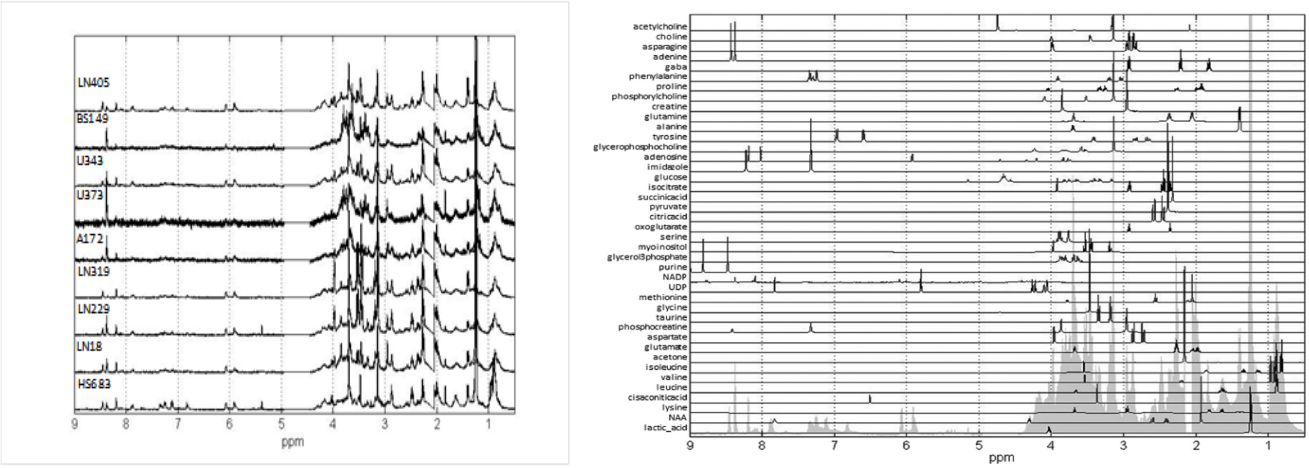


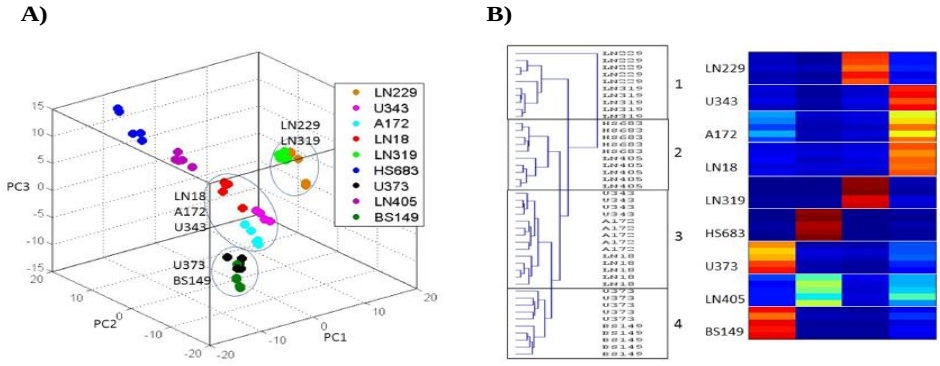
Figure 5: C/EBP-α transcript levels as obtained by RT-PCR in key glioma cell lines.

Figure 6.



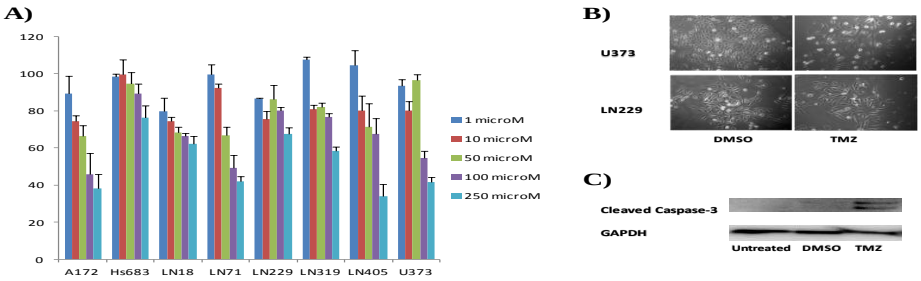
GBM cell lines NMR spectra and spectra of metabolites used for multivariate linear regression analysis of glioblastoma spectra. A) Representative NMR spectra of five biological replicates for nine glioblastoma cell lines studied in this work. The good consistency between replicates is apparent from spectral traces. B) Forty-one metabolites used in the analysis included all metabolites previously determined in NMR measurements of hydrophilic glioblastoma samples as well as samples of other cell lines. One dimensional spectra of all 41 metabolites are shown in this figure..

Figure 7.



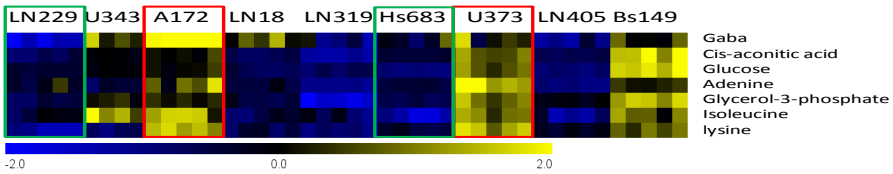
GBM clustering based on metabolic profiles. (A) Principal components analysis of spectral data for nine GBM cell lines. Metabolites were independently extracted and measured for five biological replicates corresponding to nine cell line types. The grouping of several cell types is apparent. (B) FKM and HCL clustering of spectral data. Left, HCL results for cell samples. Right, FKM determined membership values for each measurement, where red represents the membership value of 1, and dark blue corresponds to membership of 0.

Figure 8.



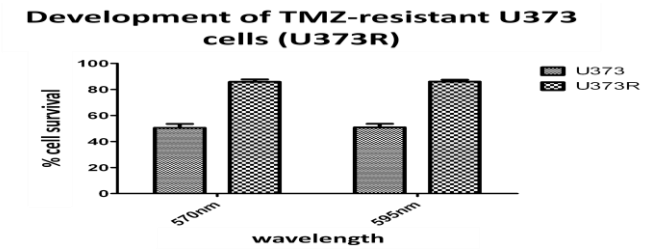
GBM cell sensitivity upon treatment with temozolomide. (A) A panel of glioma cells were treated with temozolomide (TMZ) for 72 hrs. Crystal violet staining was subsequently performed and results report % cytotoxicity of TMZ vs DMSO-treated cells. (B) Representative morphological pictures of TMZ-treated U373 and LN229 cells. (C) Cleaved Caspase-3 protein levels in U373 cells treated with medium only, DMSO only or 250 μM TMZ for 72 hrs.

Figure 9.



Differentially expressed metabolites between TMZ-sensitive and TMZ-resistant cells. Most significantly differentially concentrated metabolites in TMZ-sensitive A172/U373 (red outline) and TMZ-resistant Hs683/LN229 (green outline) cell lines. Yellow and blue colors represent strongly or weakly expressed metabolites, respectively.

Figure 10.



Generation of a TMZ-resistant cell line. Starting with U373 cells, a TMZ-sensitive cell line, a TMZ-resistant cell line was generated (U373R) by progressively increasing periodic treatments with TMZ (up to 500 μM).

Table 1.

Group of samples	Metabolites	
1 (LN229, LN319)	Taurine	Glutamine
	UDP	Glutamate
	Choline	Citric acid
	Phosphocholine	Aspartate
	Glycerophosphocholine	Asparagine
	Glycine	Methionine
	myo-Inositol	
2 (HS683, LN405)	Valine	Glutamate
	Leucine	Citric acid
	Isoleucine	Aspartate
	Alanine	Asparagine
	Lactic acid	Methionine
3 (A172, U343, LN18)	GABA	Methionine
	Proline	Citric acid
	Glutamine	Aspartate
	Glutamate	Asparagine
4 (U373, BS149)	Succinic acid	Glycerol 3-phosphate
	Serine	Glucose
	Adenine	cis-Aconitic acid
	Taurine	GABA
	Lysine	Proline
	Tyrosine	

Elevated metabolites in GBM cells. Most significantly overconcentrated metabolites for each group of GBMs when compared with the other three groups.(7)

V. RELEVANT PUBLICATIONS (2011-2013)

The following cancer-related publications were reported by the award recipient during the past two years and acknowledge the support of the Brain Tumour Foundation of Canada:

1) St-Coeur, P-D, Touaibia, M., Cuperlovic-Culf, M. and **Morin, P. Jr**. (2013) Leveraging metabolomics to assess the next generation of temozolomide-based therapeutic approaches for glioblastomas. Genomics, Proteomics & Bioinformatics. 11: 199-206. (http://www.sciencedirect.com/science/article/pii/S1672022913000508)

2) **Morin, P. Jr**., Ferguson, D., LeBlanc, L., Hébert, M., Paré, A., Jean-François, J., Surette, M., Touaibia, M. and Cuperlovic-Culf, M. (2013) NMR metabolomics analysis of the effects of 5-lipoxygenase inhibitors on metabolism in glioblastomas. J. Proteome Res. 12: 2165-2176. (http://pubs.acs.org/doi/abs/10.1021/pr400026q)

3) St-Coeur, P-D, Ferguson, D., **Morin, P. Jr.** and Touaibia, M. (2013) PF-8380 and closely related analogues synthesis, structure-activity relationship towards autotaxin inhibition and glioma cell viability. Archiv. Pharm. Chem. Life Sci. 346: 91-97. (http://onlinelibrary.wiley.com/doi/10.1002/ardp.201200395/abstract)

VI. RELEVANT CONFERENCES (2011-2013)

The following cancer-related posters/talks were presented by Dr. Morin or members of his research group during the past two years and acknowledge the support of the Brain Tumour Foundation of Canada:

1) St-Coeur, P-D, Poitras, J.J., Cuperlovic-Culf, M., Touaibia, M. and **Morin, P. Jr**. November 2013, Montréal, Canada. Temozolomide resistance in glioblastoma multiforme: metabolomics profiling as a diagnostic tool. 16th Annual Chemistry and Biochemistry Graduate Research Conference. (http://cbgrc.concordia.ca/en/presentations/)

2) St-Coeur, P-D, Poitras, J.J., Culf, A., Cuperlovic-Culf, M., Touaibia, M. and **Morin, P. Jr**. September 2013, Québec, Canada. Le glioblastome multiforme et la métabolomique: diagnostic de la résistance au témozolomide. 55e Réunion du Club de Recherches Cliniques du Québec – CRCQ 2013. (http://crcq.crc.chus.qc.ca/media/document/CRCQ_Programme_55e_reunion_annuelle.pdf)

3) Poitras, J.J., St-Coeur, P-D, Culf, A., Cuperlovic-Culf, M., Touaibia, M. and **Morin, P. Jr**. September 2013, Québec, Canada. Caractérisation et renversement de la résistance au témozolomide chez les glioblastomes. 55e Réunion du Club de Recherches Cliniques du Québec – CRCQ 2013. (http://crcq.crc.chus.qc.ca/media/document/CRCQ_Programme_55e_reunion_annuelle.pdf)

4) St-Coeur, P-D, Poitras, J.J., Cuperlovic-Culf, M, Touaibia, M and **Morin, P. Jr**. June 2013, Montréal, Canada. Diagnosing TMZ resistance in glioblastomas using metabolomics. Montreal International Symposium on Angiogenesis and Metastasis – MISAM 2013. (http://misam.mcgill.ca/)

5) **Morin, P. Jr.** June 2013, Moncton, Canada. Characterization of temozolomide resistance in glioblastomas using a metabolomics-based approach. Série de séminaires de recherche en santé Vitalité. (http://www.umoncton.ca/nouvelles/info.php?page=16&langue=0&id=12680&campus_selection=m)

6) **Morin, P. Jr**. March 2013, Moncton, Canada. Empreinte métabolique de la résistance au témozolomide chez les glioblastomes. 5e édition de la Journée de recherches interdisciplinaires en santé (JRIS) à la Faculté des sciences de la santé et des services communautaires (FSSSC) de l'Université de Moncton. (http://www.umoncton.ca/umcm-fsssc/files/umcm-fsssc/wf/Programme%202013%20-%20Francais.pdf)

7) St-Coeur, P-D, Ferguson, D, Touaibia, M. and **Morin, P. Jr**. November 2012, Fredericton, Canada. PF-8380 and closely related analogues synthesis, structure -activity relationship towards autotaxin inhibition and glioma cell viability. NBHRF 4th Annual Health Research Conference. (http://www.nbhrf.ca/)

8) Ferguson, D, Lassalle-Glaux, G, d'Eon, S, LeBlanc, L, Touaibia, M and **Morin, P. Jr.** November 2011, Halifax, Canada. 5-Lypoxygenase inhibitors: GBM's magic bullets? BHCRI 3rd Annual Cancer Conference. (http://bhcri.ca/)

9) St-Coeur, P-D, **Morin, P. Jr**. and Touaibia, M. November 2011, Halifax, Canada. Development of autotaxin inhibitors and their applications in glioblastomas. BHCRI 3rd Annual Cancer Conference. (http://bhcri.ca/)